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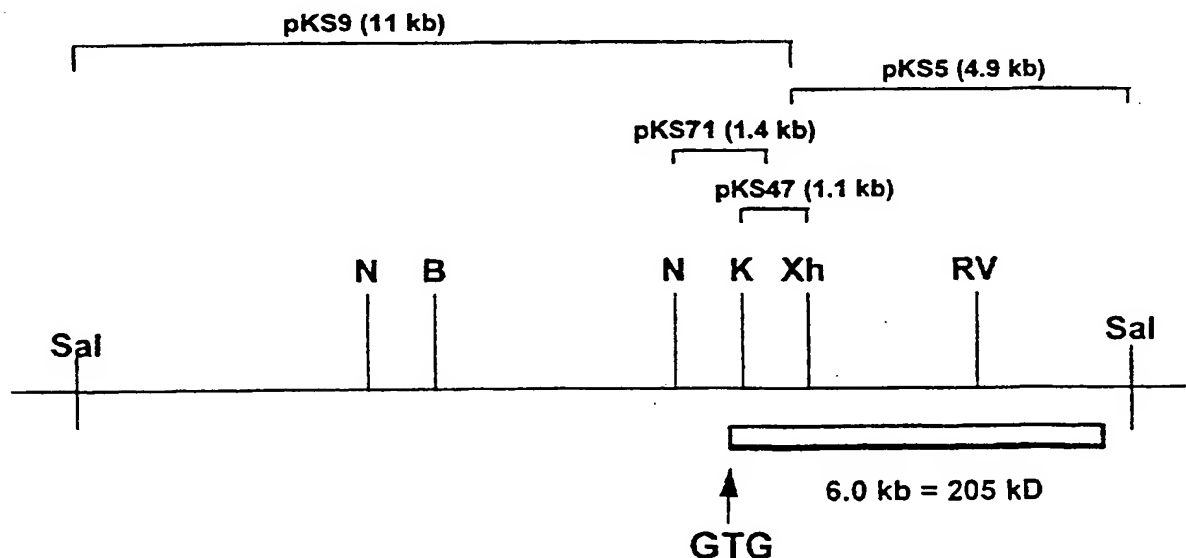
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(54) Title: HIGH MOLECULAR WEIGHT MAJOR OUTER MEMBRANE PROTEIN OF MORAXELLA



## (57) Abstract

An isolated and purified outer membrane protein of a *Moraxella* strain, particularly *M. catarrhalis*, having a molecular mass of about 200 kDa, is provided. The about 200 kDa outer membrane protein as well as nucleic acid molecules encoding the same are useful in diagnostic applications and immunogenic compositions, particularly for *in vivo* administration to a host to confer protection against disease caused by a bacterial pathogen that produces the about 200 kDa outer membrane protein or produces a protein capable of inducing antibodies in a host specifically reactive with the about 200 kDa outer membrane protein.

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TITLE OF THE INVENTION  
HIGH MOLECULAR WEIGHT MAJOR OUTER MEMBRANE  
PROTEIN OF MORAXELLA

5

FIELD OF THE INVENTION

The present invention relates to the field of immunology and is particularly concerned with outer membrane proteins from *Moraxella*, methods of production thereof, genes encoding such proteins and uses thereof.

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REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of copending United States Patent Application No. 08/478,370, filed June 7, 1995, which itself is a continuation-in-part of United States Patent Application

15 No. 08/431,718 filed May 1, 1995.

BACKGROUND OF THE INVENTION

Otitis media is the most common illness of early childhood with approximately 70% of all children suffering at least one bout of otitis media before the

20 age of seven. Chronic otitis media can lead to hearing, speech and cognitive impairment in children. It is caused by bacterial infection with *Streptococcus pneumoniae* (approximately 50%), non-typable *Haemophilus influenzae* (approximately 30%) and *Moraxella*

25 (*Branhamella*) *catarrhalis* (approximately 20%). In the United States alone, treatment of otitis media costs between one and two billion dollars per year for antibiotics and surgical procedures, such as tonsillectomies, adenoidectomies and insertion of

30 tympanostomy tubes. Because otitis media occurs at a time in life when language skills are developing at a rapid pace, developmental disabilities specifically related to learning and auditory perception have been documented in youngsters with frequent otitis media.

35

*M. catarrhalis* mainly colonizes the respiratory tract and is predominantly a mucosal pathogen. Studies using cultures of middle ear fluid obtained by

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tympanocentesis have shown that *M. catarrhalis* causes approximately 20% of cases of otitis media (ref. 1 - Throughout this application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure).

5 The incidence of otitis media caused by *M. catarrhalis* is increasing. As ways of preventing otitis media caused by pneumococcus and non-typable *H. influenzae* are developed, the relative importance of *M. catarrhalis* as a cause of otitis media can be expected to  
10 further increase.

*M. catarrhalis* is also an important cause of lower respiratory tract infections in adults, particularly in the setting of chronic bronchitis and emphysema (refs. 2, 3, 4, 5, 6, 7, and 8). *M. catarrhalis* also causes  
20 sinusitis in children and adults (refs. 9, 10, 11, 12, and 13) and occasionally causes invasive disease (refs. 14, 15, 16, 17, 18, and 19).

Like other Gram-negative bacteria, the outer membrane of *M. catarrhalis* consists of phospholipids, lipopolysaccharide (LPS), and outer membrane proteins (OMPs). Eight of the *M. catarrhalis* OMPs have been  
25 identified as major components. These are designated by letters A to H, beginning with OMP A which has a molecular mass of 98 kDa to OMP H which has a molecular  
30 mass of 21 kDa (ref. 20).

Recently, a high-molecular-weight outer membrane protein of *M. catarrhalis* was purified and characterized (ref. 21). The apparent molecular mass of this protein varies from 350 kDa to 720 kDa as judged by sodium  
35 dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This protein appears to be an oligomer of much

smaller proteins or subunits thereof of molecular mass 120 to 140 kDa and is antigenically conserved among strains of *Moraxella*.

A protein molecular mass of about 300 to 400 kDa named UspA was also reported to be present on the surface of *Moraxella* (ref. 22).

*M. catarrhalis* infection may lead to serious disease. It would be advantageous to provide other outer membrane proteins for *M. catarrhalis* and genes encoding such proteins for use as antigens in immunogenic preparations including vaccines, carriers for other antigens and immunogens and the generation of diagnostic reagents.

#### SUMMARY OF THE INVENTION

The present invention is directed towards the provision of a purified and isolated major outer membrane protein of *Moraxella catarrhalis* and other *Moraxella* strains, having an apparent molecular mass of about 200 kDa, as well as genes encoding the same.

In accordance with one aspect of the invention, there is provided an isolated and purified, outer membrane protein of a *Moraxella* strain having a molecular weight of about 200 kDa, as determined by SDS-PAGE, or a fragment or an analog thereof. The outer membrane protein may be substantially in its native conformation (so as to have substantially retained the characteristic immunogenicity of the outer membrane protein in the *Moraxella* strain) and may be isolated from a *M. catarrhalis* strain, such as from *M. catarrhalis* 4223. Such isolated and purified about 200 kDa outer membrane protein is substantially free from non-200 kDa outer membrane proteins, phospholipids and lipopolysaccharide of *Moraxella*. The about 200 kDa outer membrane protein is at least about 70 wt% pure, preferably at least about 90 wt% pure, and may be in the form of an aqueous solution thereof. Such about 200 kDa outer membrane

protein may have substantially the amino acid composition shown in Table III and a deduced amino acid sequence as shown in Figure 6 (SEQ ID No: 3).

The present invention also provides a purified and isolated nucleic acid molecule encoding an outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, as determined by SDS-PAGE, or a fragment or an analog of the outer membrane protein. The protein encoded by the nucleic acid molecule may comprise a protein containing the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-x-Gln-Gly-Ile (SEQ ID No: 5) particularly where X is Lys (SEQ ID No: 10), for *Moraxella catarrhalis* strain 4223 or containing the corresponding amino acid sequence from other *Moraxella* strains.

In a further aspect of the present invention, there is provided a purified and isolated nucleic acid molecule having a sequence selected from the group consisting of (a) a DNA sequence as set out in Figure 6 (SEQ ID Nos: 1 or 2), or the complementary sequence thereto; (b) a DNA sequence encoding an about 200 kDa protein of a strain of *Moraxella* and containing the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-x-Gln-Gly-Ile (SEQ ID No: 5), particularly where x is Lys (SEQ ID No: 10) or the complementary sequence thereto; (c) a DNA sequence encoding the deduced amino acid sequence as set out in Figure 6 (SEQ ID No: 3) or the complementary sequence thereto; and (d) a nucleotide sequence which hybridizes under stringent conditions to any one of the sequences defined in (a), (b) or (c). The nucleic acid preferably defined in (d) has at least about 90% sequence identity with any one of the sequences defined in (a), (b) or (c).

The nucleic acid molecules provided herein may be included in a vector adapted for transformation of a host. The nucleic acid molecules provided herein also

may be included in an expression vector adapted for transformation of a host along with expression means operatively coupled to the nucleic acid molecule for expression by the host of the about 200 kDa outer membrane protein of a strain of *Moraxella* or the fragment or the analog of the outer membrane protein. A transformed host containing the expression vector is included within the invention, along with a recombinant outer membrane protein or fragment or analog thereof producible by the transformed host.

The expression means may include a nucleic acid portion encoding a leader sequence for secretion from the host of the outer membrane protein or the fragment or the analog of the outer membrane protein. The expression means may include a nucleic acid portion encoding a lipidation signal for expression from the host of a lipidated form of the outer membrane protein or the fragment or analog thereof.

The present invention further includes a live vector for delivery of the outer membrane protein of the invention or a fragment or analog thereof, comprising a vector containing the nucleic acid molecule provided herein. The live vector may be selected from the group consisting of *E. coli*, *Salmonella*, BCG, adenovirus, poxvirus, vaccinia and poliovirus.

In accordance with a further aspect of the present invention, there is provided a peptide having no less than six amino acids and no more than 150 amino acids and containing an amino acid sequence corresponding to a portion only of the outer membrane protein of the invention, or a fragment or analog thereof. The peptide may be one having the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-Lys-Gln-Gly-Ile (SEQ ID No: 10) for the *Moraxella catarrhalis* 4223 strain or the amino acid sequence for the corresponding peptide for other strains of *Moraxella*.

The present invention also provides an immunogenic composition comprising an immunoeffective amount of an active component, which may be the outer membrane protein or fragment or analog thereof, nucleic acid molecules, 5 recombinant outer membrane proteins, fragments or analogs thereof, live vectors, and/or peptides, as provided herein, along with a pharmaceutically acceptable carrier therefor with the active component producing an immune response when administered to a host, which may be a 10 primate, particularly a human.

The immunogenic composition may be formulated as a vaccine for *in vivo* administration to a host to confer protection against diseases caused by a bacterial 15 pathogen that produces the about 200 kDa outer membrane protein or produces a protein capable of inducing antibodies in the host specifically reactive with the about 200 kDa outer membrane protein. In particular, the bacterial pathogen is a strain of *Moraxella*, particularly *M. catarrhalis*.

20 The immunogenic composition may be formulated as a microparticle capsule, ISCOM or liposome preparation. The immunogenic composition may be used in combination with a targeting molecule for delivery to specific cells of the immune system as to mucosal surfaces. Some 25 targeting molecules include vitamin B12 and fragments of bacterial toxins, as described in WO 92/17167 (Biotech Australia Pty. Ltd.) and monoclonal antibodies, as described in U.S. Patent No. 5,194,254 (Barber et al). The immunogenic compositions of the invention (including 30 vaccines) may further comprise at least one other immunogenic or immunostimulating material and the immunostimulating material may be at least one adjuvant.

Suitable adjuvants for use in the present invention include, (but are not limited to) aluminum phosphate, 35 aluminum hydroxide, QS21, Quil A, derivatives and components thereof, ISCOM matrix, calcium phosphate,

calcium hydroxide, zinc hydroxide, a glycolipid analog, an octadecyl ester of an amino acid, a muramyl dipeptide, polyphosphazene, ISCOPREP, DC-chol, DDBA and a lipoprotein. Advantageous combinations of adjuvants are described in copending United States Patent Application No. 08/261,194 filed June 16, 1994 and 08/483,856, filed June 7, 1995, assigned to the assignee hereof and the disclosures of which is incorporated herein by reference thereto. The invention further includes an antibody specific for the outer membrane protein provided herein producible by immunizing a host with an immunogenic composition as provided herein.

In a further aspect of the invention, there is provided a method of generating an immune response in a host comprising administering thereto an immuno-effective amount of the immunogenic composition as provided herein. The immune response may be a humoral or a cell-mediated immune response. The immune response may provide protection to the host against diseases caused by a bacterial pathogen that produces the about 200 kDa outer membrane protein or produces a protein capable of inducing antibodies in the host specifically reactive with the about 200 kDa outer membrane protein. In particular, the pathogen is a strain of *Moraxella*, including *M. catarrhalis*. Hosts in which protection against disease may be conferred include primates, including humans.

The present invention provides, in an additional aspect thereof, a method of producing a vaccine comprising administering the immunogenic composition provided herein to a test host to determine an amount and a frequency of administration of the active component to confer protection against disease caused by a bacterial pathogen that produces the about 200 kDa outer membrane protein or produces a protein capable of inducing antibodies in the host specifically reactive with the

about 200 kDa outer membrane protein, and formulating the active component in a form and amount suitable for administration to a treated host in accordance with said determined amount and frequency of administration. In particular, the pathogen is a strain of *Moraxella*, including *M. catarrhalis*. The treated host may be a human.

A further aspect of the present invention provides a method of determining the presence of nucleic acid encoding an outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, as determined by SDS-PAGE, or fragment or analog thereof, in a sample, comprising the steps of:

- (a) contacting the sample with the nucleic acid molecule provided herein to produce duplexes comprising the nucleic acid molecule and any said nucleic acid molecule encoding the outer membrane protein present in the sample and specifically hybridizable therewith; and
- (b) determining the production of the duplexes.

In yet a further aspect of the invention, there is provided a method of determining the presence of antibodies specifically reactive with outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, in a sample, comprising the steps of:

- (a) contacting the sample with the outer membrane protein as provided herein to produce complexes comprising the outer membrane protein and any said antibodies present in the sample specifically reactive therewith; and
- (b) determining production of the complexes.

In a further aspect of the invention, there is also provided a method of determining the presence of an outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, in a sample comprising the steps of:



- (a) immunizing a subject with the immunogenic composition as provided herein, to produce antibodies specific for the outer membrane protein;
- 5 (b) contacting the sample with the antibodies to produce complexes comprising any outer membrane protein present in the sample and said outer membrane protein specific antibodies; and
- 10 (c) determining production of the complexes.

The outer membrane protein may be part of a *Moraxella catarrhalis* strain.

The present invention provides, in a yet further aspect, a diagnostic kit for determining the presence of  
15 nucleic acid encoding an outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, as determined by SDS-PAGE, or fragment or analog thereof, in a sample, comprising:

- (a) the nucleic acid molecule as provided herein;
- 20 (b) means for contacting the nucleic acid with the sample to produce duplexes comprising the nucleic acid molecule and any said nucleic acid present in the sample and hybridizable with the nucleic acid molecule; and
- 25 (c) means for determining production of the duplexes.

In yet a further aspect of the invention, there is provided a diagnostic kit for determining the presence of antibodies in a sample specifically reactive with the  
30 outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, as determined by SDS-PAGE, comprising:

- (a) the outer membrane protein as provided herein;
- 35 (b) means for contacting the outer membrane protein with the sample to produce complexes

comprising the outer membrane protein and any said antibodies present in the sample; and

- (c) means for determining production of the complexes.

5 The invention also provides a diagnostic kit for detecting the presence of an outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, in a sample, comprising:

- 10 (a) an antibody specific for the about 200 kDa outer membrane protein as provided herein;
- (b) means for contacting the antibody with the sample to produce a complex comprising the outer membrane protein and outer membrane-specific antibody; and
- 15 (c) means for determining production of the complex.

In a further aspect of the invention, there is provided a method of producing an isolated and purified outer membrane protein of a strain of *Moraxella* having a

20 molecular mass of about 200 kDa, as determined by SDS-PAGE, comprising the steps of:

- (a) providing a cell mass of the *Moraxella* strain;
- (b) disrupting the cell mass to provide a cell lysate;
- 25 (c) fractionating the cell lysate to provide a fraction containing the outer membrane protein substantially free from other cell lysate components, and
- (d) recovering said outer membrane protein.

30 The bacterial strain may be *M. catarrhalis*. The cell lysate may be fractionated by gel electrophoresis.

In this application, the term "about 200 kDa protein" is used to define a family of outer membrane proteins of *Moraxella* having a molecular mass of between

35 about 160 and about 230 kDa and includes proteins having variations in their amino acid sequences including those

naturally occurring in various strains of *Moraxella*. The purified and isolated DNA molecules comprising a gene encoding the about 200 kDa protein of the present invention also include those encoding functional analogs of the about 200 kDa protein. In this application, a first protein is a "functional analog" of a second protein if the first protein is immunologically related to and/or has the same function as the second protein. The functional analog may be, for example, a fragment of the protein or a substitution, addition, deletion mutant thereof or a fusion with a second protein.

Advantages of the present invention include:

- a method for isolating purified about 200 kDa outer membrane protein of a *Moraxella* strain that produces the outer membrane protein, including *M. catarrhalis*;
- a gene encoding an about 200 kDa outer membrane protein of *M. catarrhalis*;
- an isolated and purified about 200 kDa outer membrane protein isolatable from a *Moraxella* strain; and
- diagnostic kits and immunological reagents for specific identification of *Moraxella* and hosts infected thereby.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show an analysis of *Moraxella catarrhalis* cell proteins by SDS-PAGE. The identification of the lanes and the sources of the proteins are given in Example 2 below;

Figure 2 shows a comparative analysis of cell proteins from a number of *M. catarrhalis* strains by SDS-PAGE analysis and shows the variability in the molecular weight of the about 200 kDa protein in different strains of *Moraxella*. The identification of the lanes and the sources of the proteins are given in Example 4 below;

Figure 3 shows an analysis of isolated and purified about 200 kDa outer membrane protein of *M. catarrhalis* by SDS-PAGE;

Figure 4 shows the specific recognition of about 200 kDa outer membrane protein by anti-peptide antiserum. The identification of the lanes and antiserum are given in Example 8 below;

Figure 5 shows restriction maps of clones containing a gene encoding the about 200 kDa outer membrane protein of *M. catarrhalis*. The open reading frame of the about 200 kDa outer membrane protein is indicated by the shaded box. Restriction sites are Sal: SalI, N: NcoI, B: BglII, K: KpnI, Xh: XhoI, RV: EcoRV.

Figure 6 shows the nucleotide sequence (SEQ ID No: 1 - entire sequence, SEQ ID No: 2 - coding sequence) of the gene encoding the about 200 kDa outer membrane protein of *M. catarrhalis* and the deduced amino acid sequence (SEQ ID No: 3 - identified GTG start codon, SEQ ID No: 4 - putative ATG start codon). Peptide 1 (SEQ ID No: 11) and Peptide 2 (SEQ ID No: 12) are identified by underlining;

Figure 7A is a restriction enzyme map of the gene encoding the about 200 kDa outer membrane protein of *M. catarrhalis* (SEQ ID No: 1) showing single cutting restriction enzymes;

Figure 7B is a restriction enzyme map of the gene encoding about 200 kDa outer membrane protein of *M. catarrhalis* (SEQ ID No: 1) showing double cutting restriction enzymes;

Figure 8 shows the identification of the GTG initiation codon by expressing the C-terminal truncations of the gene encoding the about 200 kDa outer membrane protein of *M. catarrhalis*. Restriction sites are N: NcoI, K: KpnI, H: HindIII, Hp: HpaI, RV: EcoRV, Sal: SalI;

Figure 9 shows the identification of the GTG initiation codon by utilization of anti-sera specific for N-terminal peptides of the about 200 kDa outer membrane protein of *M. catarrhalis*. Restriction sites are Nco:  
5 NcoI, K: KpnI, H: HindIII, RV: EcoRV, Sal: SalI;

Figure 10 shows the recognition of 200 kDa protein by anti peptide sera;

Figure 11 shows the construction of vectors for the expression of the about 200 kDa outer membrane protein of  
10 *M. catarrhalis* from *E. coli*. Nco: NcoI, Pst: PstI, Pvu: PvuII, Sca: ScaI, Sal: SalI;

Figure 12 shows the expression of N-terminal truncations of the about 200 kDa outer membrane protein of *M. catarrhalis* in *E. coli* using the bacteriophage T7  
15 promoter;

Figure 13 shows the expression of the about 200 kDa outer membrane protein of *M. catarrhalis* fused with the LacZ- $\alpha$ -peptide in *E. coli*; and

Figure 14 shows the specific identification of *M.*  
20 *catarrhalis* expressing the about 200 kDa outer membrane protein by guinea pig anti-200 kDa specific antiserum in contrast to other bacteria. Identification of the lanes and bacteria appears below.

#### 25 GENERAL DESCRIPTION OF THE INVENTION

Referring to Figure 1A and 1B and Figure 2, there is illustrated the separation of a novel outer membrane protein from a variety of strains of *M. catarrhalis* having a molecular mass about 200 kDa. The presence of  
30 this about 200 kDa protein in a variety of *M. catarrhalis* strains and, in particular, the almost-universal presence in strains isolated from patients suffering from otitis media is shown in Table I. Figure 3 shows the isolated and purified outer membrane protein.

35 Purified protein was eluted from a gel and used to raise antibodies in guinea pigs. The antibodies

specifically recognize only strains of *M. catarrhalis* which produce the outer membrane protein (Table I below).

Referring to Figure 4, there is shown the recognition of the about 200 kDa outer membrane protein by antibodies raised in guinea pigs to a synthesized peptide corresponding to an internal fragment of the about 200 kDa protein. The synthesized peptide had the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys (SEQ ID No: 6).

Referring to Figure 5, there is shown restriction maps of clones containing a gene encoding the about 200 kDa outer membrane protein. In Figure 5, the open reading frame of the about 200 kDa gene is shown as a solid box and the GTG start codon is indicated. The nucleotide sequence (SEQ ID No: 1 and 2) of the gene encoding the about 200 kDa outer membrane protein is shown in Figure 6, along with the deduced amino acid sequence (SEQ ID No: 3) of the protein. Restriction enzyme maps of the gene encoding the about 200 kDa protein are shown in Figures 7(A) and 7(B). The amino acid composition of the about 200 kDa protein is shown in Table III.

In one embodiment of the present invention, the isolated and purified about 200 kDa outer membrane protein as provided herein is useful for generating antibodies that can be used to specifically distinguish *M. catarrhalis* from other bacterial pathogens that cause otitis media and other diseases. Thus referring to Figure 14, there is illustrated an immunoblot showing the specific reactivity of a guinea pig monospecific anti-200 kDa outer membrane protein antiserum produced by immunizing mice with the purified about 200 kDa outer membrane protein as provided herein. The bacterial lysates analyzed were as follows:

<u>Lane</u>	<u>Bacterium</u>	<u>Source</u>
1	Molecular Weight Standard	
2	<i>M. catarrhalis</i> 4223	middle ear fluid
3	<i>M. catarrhalis</i> RH408	non-clumping variant of strain 4223
5		
4	<i>H. influenzae</i> , MinnA strain	meningitis isolate
5	non-typable <i>H. influenzae</i> , SB12 strain	otitis media isolate
6	non-typable <i>H. influenzae</i> , SB33 strain	otitis media isolate
7.	<i>S. pneumoniae</i> type 6	ATCC 6306
10	8. <i>S. pneumoniae</i> type 14	ATCC 6314
	9. <i>P. aeruginosa</i>	
	10. <i>E. coli</i> DH5 $\alpha$	

The results shown in Figure 14 clearly show the usefulness of outer membrane-specific antisera as provided herein to distinguish between bacterial pathogens that produce diseases with similar clinical symptoms.

In accordance with another aspect of the present invention, there is provided a vaccine against *Moraxella*, comprising an immunogenically-effective amount of the outer membrane protein as provided herein and a physiologically-acceptable carrier therefor. The outer membrane protein provided herein also may be used as a carrier protein for hapten, polysaccharides or peptides to make a conjugate vaccine against antigenic determinants unrelated to the about 200 kDa outer membrane protein.

The about 200 kDa outer membrane protein provided herein is useful as a diagnostic reagent, as an antigen for the generation of anti-outer membrane protein antibodies, or as an antigen for vaccination against the diseases caused by species of *Moraxella* or for detecting infection by *Moraxella*.

In additional embodiments of the present invention, the about 200 kDa outer membrane protein as provided herein may be used as a carrier molecule to prepare chimeric molecules and conjugate vaccines (including glycoconjugates) against pathogenic bacteria, including encapsulated bacteria. Thus, for example, glycoconjugates of the present invention may be used to confer protection against disease and infection caused by any bacteria having polysaccharide antigens including lipooligosaccharides (LOS) and polyribosylphosphate (PRP). Such bacterial pathogens may include, for example, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Escherichia coli*, *Neisseria meningitidis*, *Salmonella typhi*, *Streptococcus mutants*, *Cryptococcus neoformans*, *Klebsiella*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Particular antigens which can be conjugated to outer membrane protein and methods to achieve such conjugations are described in published PCT application WO 94/12641, assigned to the assignee hereof and the disclosure of which is hereby incorporated by reference thereto.

In another embodiment, the carrier function of the outer membrane protein may be used, for example, to induce immunity toward abnormal polysaccharides of tumor cells, or to produce anti-tumor antibodies that can be conjugated to chemotherapeutic or bioactive agents.

The present invention extends to the use of the nucleic acid molecules and proteins provided herein as a medicament and in the manufacture of a medicament for the treatment of *Moraxella* infections.

In a particular embodiment of the invention, there is provided a recombinant about 200 kDa outer membrane protein of *Moraxella* or fragment or analog thereof or a fusion protein producible by a transformed host containing at least a portion of the gene encoding the about 200 kDa protein. Referring to Figure 11, there is



shown recombinant vectors for the production of such proteins. In Figure 11, the filled boxes show 1.9 kb and 4.8 kb C-terminal regions of 200 kD protein gene, that were inserted into a vector, pT7-7, under the control of the bacteriophage T7 promoter. The small open boxes show seven N-terminal amino acids from the vector in the same reading frame. The shaded box shows 5.5 kb C-terminal region of 200 kD protein, which contained ATG codon very close to the N-terminus. This gene fragment was fused to lacZ  $\alpha$  peptide gene (shown in filled box) under the control of lacZ promoter. The full-length gene, that starts from GTG, is shown in a hatched box.

Referring to Figure 12, there is shown the expression of N-terminal truncations of the about 200 kDa protein in *E. coli*. *E. coli* strain, BL21(DE3)/pLyss, carrying plasmid, pKS94, was grown in LB broth containing 100  $\mu$ g/ml ampicillin to the early log phase and then IPTG was added. After culturing for 2 more hours, the bacteria were harvested and lysed. The lysates were assayed on Western blot using anti-200 kD protein guinea pig serum as a first antibody. Other procedures were as in Fig. 5. Lane 1: prestained molecular weight marker, Lane 2: BL21(DE3)/pLyss carrying pT7-7 with an incorrect insert. Lane 3: BL21(DE3)/pLyss carrying pKS94.

Referring to Figure 13, there is shown the expression of fusion protein comprising the  $\beta$ -galactosidase  $\alpha$  peptide and a portion of the about 200 kDa protein in *E. coli*. *E. coli* strain, DH5 $\alpha$ , carried pKS140. The plasmid pKS140 carried the C-terminal 5.5 kb fragment of 200 kD protein gene after a N-terminal portion of LacZ- $\alpha$ -peptide in the same reading frame. The *E. coli* strain was grown to the stationary phase, harvested and then lysed. The lysate was assayed by Western blotting. Lane 1: prestained molecular weight marker, Lane 2: DH5 $\alpha$  carrying pKS140 (total protein, 0.5

μg), Lane 3: sonicate of *M. catarrhalis*, strain 4223 (total protein, 10 μg).

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis, treatment of *Moraxella* infections, and in the generation of immunological reagents. A further non-limiting discussion of such uses is further presented below.

#### 1. Vaccine Preparation and Use

Immunogenic compositions, including those suitable to be used as vaccines, may be prepared from the about 200 kDa outer membrane protein as disclosed herein, as well as immunological fragments and fusions thereof, which may be purified from the bacteria or which may be produced recombinantly. The vaccine elicits an immune response in a subject which produces antibodies, including anti-200 kDa outer membrane protein antibodies and antibodies that are opsonizing or bactericidal. Should the vaccinated subject be challenged by *Moraxella* or other bacteria that produce proteins capable of producing antibodies that specifically recognize 200 kDa outer membrane protein, the antibodies bind to and inactivate the bacterium. Furthermore, opsonizing or bactericidal anti-200 kDa outer membrane protein antibodies may also provide protection by alternative mechanisms.

Immunogenic compositions including vaccines may be prepared as injectables, as liquid solutions or emulsions. The about 200 kDa outer membrane protein may be mixed with pharmaceutically acceptable excipients which are compatible with the about 200 kDa outer membrane protein. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or

emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously or intramuscularly. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 1 to 95% of the about 200 kDa outer membrane protein. The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the about 200 kDa outer membrane protein. Suitable regimes for initial administration and booster doses are also variable, but may include an initial

administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host.

The immunogenic preparations including vaccines may  
5 comprise as the immunostimulating material a nucleotide vector comprising at least a portion of the gene encoding the about 200 kDa protein, or the at least a portion of the gene may be used directly for immunization.

The concentration of the about 200 kDa outer  
10 membrane antigen in an immunogenic composition according to the invention is in general about 1 to 95%. A vaccine which contains antigenic material of only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and  
15 also belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

Immunogenicity can be significantly improved if the  
20 antigens are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution in phosphate-buffered saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen  
25 locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune  
30 responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Intrinsic adjuvants, such as lipopolysaccharides, normally are the components of the  
35 killed or attenuated bacteria used as vaccines. Extrinsic adjuvants are immunomodulators which are

typically non-covalently linked to antigens and are formulated to enhance the host immune responses. Thus, adjuvants have been identified that enhance the immune response to antigens delivered parenterally. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diphtheria and tetanus toxoids is well established and a HBsAg vaccine has been adjuvanted with alum. While the usefulness of alum is well established for some applications, it has limitations. For example, alum is ineffective for influenza vaccination and inconsistently elicits a cell mediated immune response.

A wide range of extrinsic adjuvants can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens (immune stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as lipid A, and liposomes.

To efficiently induce humoral immune responses (HIR) and cell-mediated immunity (CMI), immunogens are typically emulsified in adjuvants. Many adjuvants are toxic, inducing granulomas, acute and chronic inflammations (Freund's complete adjuvant) FCA, cytotoxicity (saponins and Pluronic polymers) and pyrogenicity, arthritis and anterior uveitis (LPS and MDP). Although FCA is an excellent adjuvant and widely used in research, it is not licensed for use in human or veterinary vaccines because of its toxicity.

Desirable characteristics of ideal adjuvants include:

- (1) lack of toxicity;
- (2) ability to stimulate a long-lasting immune response;
- 5 (3) simplicity of manufacture and stability in long-term storage;
- (4) ability to elicit both CMI and HIR to antigens administered by various routes, if required;
- (5) synergy with other adjuvants;
- 10 (6) capability of selectively interacting with populations of antigen presenting cells (APC);
- (7) ability to specifically elicit appropriate  $T_H1$  or  $T_H2$  cell-specific immune responses; and
- (8) ability to selectively increase appropriate antibody
- 15 isotype levels (for example, IgA) against antigens.

U.S. Patent No. 4,855,283 granted to Lockhoff et al on August 8, 1989 which is incorporated herein by reference thereto, teaches glycolipid analogues including N-glycosylamides, N-glycosylureas and N-glycosylcarbamates, each of which is substituted in the sugar residue by an amino acid, as immuno-modulators or adjuvants. Thus, Lockhoff et al. (US Patent No. 4,855,283 and ref. 27) reported that N-glycolipid analogs displaying structural similarities to the naturally-occurring glycolipids, such as glycosphospholipids and glycolycerolipids, are capable of eliciting strong immune responses in both herpes simplex virus vaccine and pseudorabies virus vaccine. Some glycolipids have been synthesized from long chain-alkylamines and fatty acids that are linked directly with the sugars through the anomeric carbon atom, to mimic the functions of the naturally occurring lipid residues.

U.S. Patent No. 4,258,029 granted to Moloney, assigned to the assignee hereof and incorporated herein by reference thereto, teaches that octadecyl tyrosine hydrochloride (OTH) functioned as an adjuvant when

complexed with tetanus toxoid and formalin inactivated type I, II and III poliomyelitis virus vaccine. Also, Nixon-George et al. (ref. 24), reported that octadecyl esters of aromatic amino acids complexed with a recombinant hepatitis B surface antigen, enhanced the host immune responses against hepatitis B virus.

Lipidation of synthetic peptides has also been used to increase their immunogenicity. Thus, Wiesmuller (ref. 25) describes a peptide with a sequence homologous to a foot-and-mouth disease viral protein coupled to an adjuvant tripalmityl-S-glyceryl-cysteinylserylserine, being a synthetic analogue of the N-terminal part of the lipoprotein from Gram negative bacteria. Furthermore, Deres et al. (ref. 26) reported *in vivo* priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine which comprised of modified synthetic peptides derived from influenza virus nucleoprotein by linkage to a lipopeptide, N-palmityl-S-[2,3-bis(palmitylxy)-(2RS)-propyl-[R]-cysteine (TPC).

## 2. Immunoassays

The about 200 kDa outer membrane protein of the present invention is useful as an immunogen for the generation of anti-200 kDa outer membrane protein antibodies, as an antigen in immunoassays including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art for the detection of anti-bacterial, anti-Moraxella, and anti-200 kDa outer membrane protein antibodies. In ELISA assays, the about 200 kDa outer membrane protein is immobilized onto a selected surface, for example, a surface capable of binding proteins such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed about 200 kDa outer membrane protein, a nonspecific protein such as a solution of bovine serum albumin (BSA) that is known to be antigenically neutral

with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific  
5 bindings of antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials, to be tested in a manner conducive to immune complex (antigen/antibody) formation. This may include diluting  
10 the sample with diluents, such as solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from 2 to 4 hours, at temperatures such as of the order of about 20° to 37°C. Following incubation,  
15 the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween or a borate buffer. Following formation of specific immunocomplexes between the test sample and the bound  
20 about 200 kDa outer membrane protein, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human  
25 origin, the second antibody is an antibody having specificity for human immunoglobulins and in general IgG. To provide detecting means, the second antibody may have an associated activity such as an enzymatic activity that will generate, for example, a colour development upon  
30 incubating with an appropriate chromogenic substrate. Quantification may then be achieved by measuring the degree of colour generation using, for example, a visible spectrophotometer.

### 3. Use of Sequences as Hybridization Probes

35 The nucleotide sequences of the present invention, comprising the sequence of the about 200 kDa protein



gene, now allow for the identification and cloning of the about 200 kDa protein gene from any species of *Moraxella*.

The nucleotide sequences comprising the sequence of the about 200 kDa protein gene of the present invention are useful for their ability to selectively form duplex molecules with complementary stretches of other about 200 kDa protein genes. Depending on the application, a variety of hybridization conditions may be employed to achieve varying degrees of selectivity of the probe toward the other genes. For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of between about 50°C to 70°C. For some applications, less stringent hybridization conditions are required such as 0.15 M to 0.9 M salt, at temperatures ranging from between about 20°C to 55°C. Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be a method of choice depending on the desired results. In general, convenient hybridization temperatures in the presence of 50% formamide are: 42°C for a probe which is 95 to 100% homologous to the target fragment, 37°C for 90 to 95% homology and 32°C for 85 to 90% homology.

In a clinical diagnostic embodiment, the nucleic acid sequences of the about 200 kDa protein genes of the present invention may be used in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin and digoxigenin-labelling, which are capable of providing a detectable signal. In some diagnostic embodiments, an

enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of a radioactive tag may be used. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with samples containing about 200 kDa protein gene sequences.

The nucleic acid sequences of the about 200 kDa protein genes of the present invention are useful as hybridization probes in solution hybridizations and in embodiments employing solid-phase procedures. In embodiments involving solid-phase procedures, the test DNA (or RNA) from samples, such as clinical samples, including exudates, body fluids (e. g., serum, amniotic fluid, middle ear effusion, sputum, bronchoalveolar lavage fluid) or even tissues, is adsorbed or otherwise affixed to a selected matrix or surface. The fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes comprising the nucleic acid sequences of the about 200 kDa protein encoding genes or fragments or analogs thereof of the present invention under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required depending on, for example, the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe etc. Following washing of the hybridization surface so as to remove non-specifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label. It is preferred to select nucleic acid sequence portions which are conserved among species of *Moraxella*. The selected probe may be at least 18bp and may be in the range of about 30 to 90 bp.

#### 4. Expression of the about 200 kDa Protein Gene

Plasmid vectors containing replicon and control sequences which are derived from species compatible with

the host cell may be used for the expression of the genes encoding the about 200 kDa protein in expression systems. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* may be transformed using pBR322 which contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The plasmids or phage, must also contain, or be modified to contain, promoters which can be used by the host cell for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host can be used as a transforming vector in connection with these hosts. For example, the phage in lambda GEM<sup>TM</sup>-11 may be utilized in making recombinant phage vectors which can be used to transform host cells, such as *E. coli* LE392.

Promoters commonly used in recombinant DNA construction include the  $\beta$ -lactamase (penicillinase) and lactose promoter systems and other microbial promoters, such as the T7 promoter system as described in U.S. Patent No. 4,952,496. Details concerning the nucleotide sequences of promoters are known, enabling a skilled worker to ligate them functionally with genes. The particular promoter used will generally be a matter of choice depending upon the desired results. Hosts that are appropriate for expression of the about 200 kDa protein genes, fragments, analogs or variants thereof, may include *E. coli*, *Bacillus* species, *Haemophilus*, fungi, yeast, *Bordetella*, or the baculovirus expression system may be used.

In accordance with this invention, it is preferred to make the protein by recombinant methods, particularly when the naturally occurring about 200 kDa protein as purified from a culture of a species of *Moraxella* may include trace amounts of toxic materials or other

contaminants. This problem can be avoided by using recombinantly produced protein in heterologous systems which can be isolated from the host in a manner to minimize contaminants in the purified material.

5 Particularly desirable hosts for expression in this regard include Gram positive bacteria which do not have LPS and are, therefore, endotoxin free. Such hosts include species of *Bacillus* and may be particularly useful for the production of non-pyrogenic about 200 kDa

10 protein, fragments or analogs thereof.

#### BIOLOGICAL DEPOSITS

Certain plasmids that contain portions of the gene having the open reading frame of the gene encoding the

15 about 200 kDa outer membrane protein of *M. catarrhalis* strain 4223 that are described and referred to herein have been deposited with the America Type Culture Collection (ATCC) located at 12301 Parklawn Drive, Rockville, Maryland, 20852, U.S.A., pursuant to the

20 Budapest Treaty and pursuant to 37 CFR 1.808 and prior to the filing of this application. The identifications of the respective portions of the gene present in these plasmids are shown in Figure 5.

Samples of the deposited plasmids will become

25 available to the public upon grant of a patent based upon this United States patent application. The invention described and claimed herein is not to be limited in scope by plasmids deposited, since the deposited embodiment is intended only as an illustration of the

30 invention. Any equivalent or similar plasmids that encode similar or equivalent antigens as described in this application are within the scope of the invention.

	<u>Plasmid</u>	<u>ATCC Designation</u>	<u>Date Deposited</u>
	pKS47	97,111	April 7, 1995
35	pKS5	97,110	April 7, 1995
	pKS9	97,114	April 18, 1995

### EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These  
5 Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been  
10 employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the  
15 scientific literature and are well within the ability of those skilled in the art.

#### Example 1

This Example illustrates the generation of a non-clumping strain (RH408) of *M. catarrhalis*.

20. *M. catarrhalis* strain 4223, a clumping strain (a common property of *Moraxella* strains), was inoculated into several flasks containing 20 mL of brain heat infusion (BHI) broth, and the cultures were incubated with shaking (170 rpm) overnight at 37°C. Five mL of  
25 each overnight culture were transferred to five individual 1 mL tubes, and were left sitting undisturbed at room temperature for 3 to 8 hours, to allow bacteria to sediment. One hundred  $\mu$ L of the cleared upper phase of each culture were used to inoculate 25 mL of BHI broth  
30 and cultures were incubated overnight at 37°C, as described above. This passaging was repeated six times, using 25  $\mu$ L of cleared culture to inoculate 25 mL of BHI for each overnight culture. Non-clumping bacterial cultures were identified by measuring the absorbency  $A_{578}$   
35 at intervals over a 3 hour time period, in order to compare the sedimentation rates of the passaged strains

to that of the original *M. catarrhalis* strain 4223 culture. Non-clumping mutants, including *M. catarrhalis* RH408, did not aggregate during the three hour time period. On BHI agar plates, strain RH408 had a colony morphology typical for all non-clumping strains. Strain RH408 was previously deposited in connection of United States Application No. 08/328,589 at the ATCC under the Budapest Treaty on December 13, 1994 with Accession No. 55637.

10 Example 2

This Example illustrates the identification of the about 200 kDa outer membrane protein of *Moraxella catarrhalis*.

*M. catarrhalis* strains 4223, RH408, 5191, 8185, M2, M5, ATCC 25240, 3, 56, 135, 585 were grown in brain heart infusion (BHI) broth. The culture was incubated overnight with aeration at 37°C.

*M. catarrhalis* cells were sonicated and total protein was determined using the BCA assay system (Pierce, Rockford, IL). Ten µg of total protein were mixed with the SDS-PAGE sample buffer containing 0.3M Tris-HCl (pH 8.0), 50% glycerol, 10% SDS, 20% β-mercaptoethanol and 0.01% bromophenol blue, boiled for 5 minutes and loaded on each lane of SDS-PAGE gel (0.75 mm thick, 7.5% acrylamide). The gels were run at 200 V for 1 hour. Proteins were visualized by staining gels with a solution containing 0.13% Coomassie brilliant blue, 10% acetic acid and 45% methanol. Excess stain was removed with a destaining solution of 5% ethanol and 7.5% acetic acid.

The various *Moraxella* proteins separated by this procedure are shown in Figures 1A and 1B. The *M. catarrhalis* strains tested were as follows:

**Figure 1A**

	<u>Lane</u>	<u>Bacterial Strain</u>	<u>Source</u>
	1.	Molecular Weight Standards	
	2.	<i>E. coli</i>	
5	3.	No sample	
	4.	<i>M. catarrhalis</i> 4223	middle ear fluid
	5	<i>M. catarrhalis</i> RH408	non-clumping variant of 4223
	6.	<i>M. catarrhalis</i> 5191	middle ear fluid
10	7.	<i>M. catarrhalis</i> 8185	nasopharynx
	8.	<i>M. catarrhalis</i> M2	sputum
	9.	<i>M. catarrhalis</i> M5	sputum
	10.	<i>M. catarrhalis</i> 25240	ATCC 25240

15 **Figure 1B**

	<u>Lane</u>	<u>Bacterial Strain</u>	<u>Source</u>
	1.	<i>E. coli</i>	
	2.	No sample	
	3.	Molecular Weight Size Markers	
20	4.	<i>M. catarrhalis</i> 4223	middle ear fluid
	5	<i>M. catarrhalis</i> RH408	non-clumping variant of 4223
	6.	<i>M. catarrhalis</i> 3	sputum
	7.	<i>M. catarrhalis</i> 56	sputum
25	8.	<i>M. catarrhalis</i> 135	middle ear fluid
	9.	<i>M. catarrhalis</i> 585	Blood

30 The about 200 kDa outer membrane protein was clearly seen in all otitis media strains (*M. catarrhalis* 4223, 5191, 135); in one strain isolated from the nasopharynx (8185), and in one strain isolated from sputum (M2). However, the about 200 kDa protein was not detected in three isolates from sputum (3, 56 and M5) and in one strain

with unknown origin (ATCC 25240). A very narrow band was found in an isolate from blood of a bacteremia patient (585) and this band was recognized by an anti-200 kDa specific guinea pig serum on an immunoblot. Strain  
 5 RH408 is a non-clumping spontaneous mutant isolated from strain 4223 (see Example 1) and was found to not express the about 200 kDa protein.

When gels were run longer, they showed heterogeneity in the apparent molecular masses of the about 200 kDa  
 10 outer membrane protein in different strains of *M. catarrhalis* (Fig 2). In Figure 2 the strains analyzed were as follows:

	<u>Lane</u>	<u>Strain</u>	<u>Source</u>
15	1.	Molecular Weight Size Markers	
	2.	<i>M. catarrhalis</i> H04	middle ear fluid
	3	<i>M. catarrhalis</i> H12	middle ear fluid
	4.	<i>M. catarrhalis</i> PO34	middle ear fluid
	5.	<i>M. catarrhalis</i> PO51	middle ear fluid
20	6.	<i>M. catarrhalis</i> E-07	middle ear fluid
	7.	<i>M. catarrhalis</i> E-22	middle ear fluid
	8.	<i>M. catarrhalis</i> E-23	middle ear fluid
	9.	<i>M. catarrhalis</i> RH 4223	middle ear fluid
25	10.	<i>M. catarrhalis</i> RH 408	Non-clumping variant of 4223

The strain H12 (lane 3) was a natural isolate from middle ear fluid, but did not produce the about 200 kDa protein.

There may be at least three different sizes of protein in the about 200 kDa range. However, antibodies  
 30 raised against the about 200 kDa outer membrane protein from one strain of *M. catarrhalis* (4223) did recognize all about 200 kDa proteins tested, present in different strains of *M. catarrhalis*. It is possible, however, that in particular immunogenic compositions, for example, as  
 35 a vaccine and in particular diagnostic embodiments, that



the about 200 kDa outer membrane protein from a variety of *M. catarrhalis* isolates (including immunogenically diverse isolates) may be required.

Example 3

5           This Example illustrates the detection of antibodies specific for the about 200 kDa outer membrane protein in a serum obtained from a convalescent patient having recovered from otitis media due to *M. catarrhalis*.

          After separation by SDS-PAGE, bacterial proteins  
10 were transferred from polyacrylamide gels to prepared PVDF (polyvinylidene fluoride; Millipore) membranes at a constant voltage of 70 V for 1.5 h in a buffer system consisting of 3 g Tris, 14.4 g glycine and 200 ml methanol per liter at 4°C. Membranes with transferred  
15 proteins were blocked with Blocking Reagent (from Boehringer Mannheim) diluted in TBS (0.1M Tris, 0.15M NaCl) at room temperature for 30 min. Blots were exposed to convalescent antiserum diluted 1:500 in Blocking Reagent/TBS with 0.1% Tween 20 for 2 hours at room  
20 temperature. This patient had otitis media and the *M. catarrhalis* strain isolated from the patient's ear fluid was *M. catarrhalis* CJ7. Blots were then washed 2 times in Blocking Reagent/TBS with Tween at 15 min per wash. The reporter conjugate, horseradish peroxidase (HRP)  
25 conjugated to protein G, was diluted 1:4000 with Blocking Reagent/TBS with Tween and used to immerse the washed membranes for 30 min at room temperature. Blots were washed twice as above, followed by a TBS wash. Bound antibodies were detected using the LumiGlo (Kirkegaard  
30 and Perry) chemiluminescent detection system as described by the manufacturer. Treated blots were exposed to X-ray film. Antibodies were detected in this convalescent serum that reacted with the about 200 kDa outer membrane protein of *M. catarrhalis* CJ7. These results indicate  
35 that the about 200 kDa outer membrane protein is an immunogenic protein of *M. catarrhalis* to which an immune

response is elicited during a natural infection by *M. catarrhalis*.

Example 4

This Example illustrates the isolation and  
5 purification of the about 200 kDa outer membrane protein.

*M. catarrhalis* 4223 cells were harvested by centrifugation at 2,000 rpm for 10 min and frozen. The frozen cells were thawed, resuspended in 20 mM sodium phosphate buffer (pH 7.2) and sonicated until the cells  
10 were disrupted. The frozen-thawed cells were also lysed in 20 mM Tris buffer (pH 8) containing 4% SDS and 0.2 mM EDTA by boiling for 5 min to produce a cell lysate. The cell sonicates and cell lysates were suspended in a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample  
15 buffer, boiled for 5 min and separated by SDS-PAGE on a gel (1.5 mm thick, 7.5% acrylamide). The estimated position of the about 200 kDa protein on the gel was cut out and the protein extracted from the gel by electroelution using the same buffer as the SDS-PAGE  
20 running buffer. The isolated about 200 kDa outer membrane protein was shown to be a homogeneous, single band by SDS-PAGE as seen in Figure 3. The samples analyzed in Figure 3 are as follows:

25	<u>Lane</u>	<u>Sample</u>
	1.	Molecular Weight Size Markers
	2.	Isolated and purified 200 kDa outer membrane protein

30 The isolated and purified 200 kDa outer membrane protein of *M. catarrhalis* shown in Figure 3 has a purity of at least 70%. Purified about 200 kDa outer membrane protein preparations of at least 95% could be readily achieved.

Example 5

This Example illustrates the immunization of guinea pigs with purified about 200 kDa protein from *M. catarrhalis*.

5        Approximately 30 to 40  $\mu$ g of the about 200 kDa protein, which was isolated from *M. catarrhalis* strain 4223 by electroelution, were mixed with Freund's complete adjuvant (FCA) and subcutaneously injected into guinea pigs. After two weeks, the animals were boosted with  
10       about the same amount of the about 200 kDa protein in incomplete Freund's adjuvant (IFA). Two weeks later, blood was collected from the guinea pigs and antisera were obtained.

One antiserum was examined on Western blot for its  
15       reactivity with the about 200 kDa protein present in 54 different strains of *M. catarrhalis*, which were isolated in different geographical locations throughout the world (Canada, U.S. and Finland) (see Table 1 below). The about 200 kDa protein was recognized by the antiserum in  
20       all strains, in which the presence of the about 200 kDa protein band was detected on SDS-PAGE gels stained with Coomassie Blue. These results indicate that common epitopes of the about 200 kDa protein were present in all  
25       *M. catarrhalis* strains, which possessed this protein. As stated earlier, this protein is not present in all *M. catarrhalis* strains, but almost all strains, which were isolated from middle ear fluids from otitis media patients, did possess this protein (Table 1).

Example 6

30       This Example illustrates the specific recognition of *M. catarrhalis* strain 4223 with anti-200 kDa protein guinea pig serum by ELISA assay (see Table 2 below).

*M. catarrhalis* strains 4223, RH408 (200 kDa protein negative mutant) and H-12 were cultured in 60 mL of BHI  
35       broth overnight. *E. coli* strain BL21 (DE3) was cultured in 60 mL of broth overnight. The cultures were split

into three tubes and centrifuged. *M. catarrhalis* strain 4223 was centrifuged at 1,500 rpm for 10 min., H-12 at 2,000 rpm for 10 min., and RH408 and *E. coli* BL21 (DE3) at 3,000 rpm for 15 min. The pellet in one tube was  
5 suspended in 20 ml of Dulbecco's phosphate buffered saline (D-PBS) and diluted to 1/500 with coating buffer (0.05M carbonate/bicarbonate buffer) pH 9.6. One hundred  $\mu$ L of the bacteria suspension were placed in each well and incubated for 1 hour at room temperature. One  
10 hundred  $\mu$ L of 0.2% glutaraldehyde was added to each well and incubated at room temperature for 10 min. to fix the cells on the well. The wells were washed with PBS containing 0.1% Tween 20 and 0.1% BSA (washing buffer), and then blocked with PBS containing 0.1% BSA for 30 min.  
15 at room temperature. After washing 5 times for 10 seconds with the washing buffer, serial dilutions of guinea pig antiserum with the washing buffer were added to the wells and incubation at room temperature was continued for 60 min. After washing, goat anti-guinea  
20 pig IgG conjugated with horseradish peroxidase was added to each well at the dilution of 1/20,000. After incubation at room temperature for 60 minutes, the wells were washed and then color reaction was developed using 3,3'-5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide.  
25 The ELISA plate wells were also coated with sonicates containing 10  $\mu$ g/mL of total proteins in the coating buffer, blocked without the fixation process and then assayed as described above.

The results shown in Table 2 indicate that the about  
30 200 kDa outer membrane protein specific guinea pig antiserum specifically recognizes strains of *M. catarrhalis* which produce the about 200 kDa protein. The ability of the antiserum to recognize whole cells indicates that the protein is present on the surface of  
35 the bacterial cells.

Example 7

This Example describes the determination of an internal amino acid sequence of the 200 kDa outer membrane protein.

5       The about 200 kDa outer membrane protein was isolated from *M. catarrhalis* 4223 by electroelution as described above. The protein was subjected to CNBr degradation, the proteolytic digests subjected to SDS-PAGE and transferred onto PVDF membrane. A peptide band  
10       migrating at a position corresponding to approximately 40 kDa was cut out from the membrane and its N-terminal amino acid sequence was determined. In another experiment, the CNBr degradation products of the about 200 kDa protein were subjected to a direct determination  
15       of N-terminal amino acid sequencing without separating by SDS-PAGE. Both analyses gave an identical, N-terminal sequence of 20 amino acids with one unidentified amino acid at the 17th position. The internal sequence of the 200 kDa outer membrane protein was:

20       NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-X-Gln-Gly-Ile (SEQ ID No: 5).

Example 8

This Example describes the immunization of guinea pigs with a peptide corresponding to an internal fragment  
25       of the about 200 kDa outer membrane protein and the analysis of the antiserum generated.

Based upon the determination of the amino acid sequence of an internal fragment of the about 200 kDa outer membrane protein as described above, a 16 amino  
30       acid long peptide of sequence:

NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys (SEQ ID No: 6)

was synthesized using standard procedures. This 16-mer peptide was conjugated to KLH using Imject Maleimide  
35       Activated KLH (Pierce, Rockford, IL ) and approximately 500 µg of the conjugate was injected into guinea pigs

using the same immunization and boosting schedule as described above. The guinea pig anti-serum raised against the 16-mer internal amino acid sequence (SEQ ID No: 6) was examined by immunoblot analysis and found to specifically recognize 200 kDa outer membrane protein in cell sonicates of *M. catarrhalis* 4223. The results are shown in Figure 4 and indicate that the anti-peptide guinea pig antiserum specifically recognizes the 200 kDa protein of *M. catarrhalis* 4223. The samples analyzed in Figure 4 were as follows:

	<u>Lane</u>	<u>Sample</u>	<u>Antiserum</u>
	1.	Molecular Weight Markers	
15	2.	Purified 200 kDa outer membrane protein	Anti-200 kDa protein
	3.	<i>M. catarrhalis</i> cell sonicate	Anti-peptide 1:5000
	4	<i>M. catarrhalis</i> cell sonicate	Anti-peptide 1:1000
	5.	<i>M. catarrhalis</i> cell sonicate	Anti-peptide 1:500
20	6.	<i>M. catarrhalis</i> cell sonicate	Pre-immune serum

The results obtained confirm that the peptide corresponding to SEQ ID Nos: 5 and 6 are derived from the 200 kDa outer membrane protein.

#### 25 Example 9

This Example describes the preparation of a *M. catarrhalis* genomic library.

Chromosomal DNA was isolated as follows:

An *M. catarrhalis* cell pellet was resuspended in 20 mL of Tris-EDTA (TE) buffer, pH 7.5. Pronase (final concentration 500 µg/mL) and SDS (final concentration 1%) were added and the suspension was incubated at 37°C for 2 hours. DNA was isolated by sequential extractions once with phenol, twice with phenol-chloroform (1:1), and once with chloroform-isoamyl alcohol (24:1). Extracted DNA was dialyzed against 1M NaCl at 4°C for 4 hours. This

was followed by dialysis against TE buffer, pH 7.5, at 4°C for 48 hours (3 buffer changes). DNA was ethanol precipitated from the dialysate. Large-size DNA was collected by spooling on a glass rod, air dried and dissolved in 3 mL water. Small scale *Sau3A* (New England BioLabs) restriction digests of chromosomal DNA (final volume 10  $\mu$ l) were done to establish conditions required to obtain maximal amounts of chromosomal DNA with a size range of 15 - 23 kb. Large scale digests were prepared once the optimal digestion conditions were determined. The large scale digests consisted of 50  $\mu$ L of chromosomal DNA (290  $\mu$ g/mL), 33  $\mu$ L water, 10  $\mu$ L *Sau3A* buffer (New England BioLabs), 1  $\mu$ L BSA (10 mg/ml, New England BioLabs) and 6.3  $\mu$ L *Sau3A* (0.04 U/ $\mu$ L), and were incubated at 37°C for 15 min. Reactions were stopped by the addition of 10  $\mu$ L 10X loading buffer (100 mM Tris-HCl pH 8, 10 mM EDTA, 0.1% bromophenol blue, 50% glycerol). Digested DNA was applied to 0.5% agarose gels (prepared in Tris-acetate-EDTA (TAE)) and separated according to size at 50 V for 6 hours. The region of the gel encompassing DNA of size 15-23 kb was cut from the gel and placed in dialysis tubing (BRL) with 3 mL of TAE. DNA was electroeluted from the gel-slice overnight at a field strength of 1 V/cm. Electroeluted DNA in TAE was extracted once with phenol, once with phenol-chloroform (1:1), and precipitated with ethanol. The dried DNA pellet was dissolved in 5  $\mu$ L water. Size-fractionated chromosomal DNA was ligated with *Bam*HI cut EMBL3 arms (Promega) using T4 DNA ligase in a final volume of 9  $\mu$ L. The entire ligation reaction was packaged into phage  $\lambda$  using a commercial packaging kit (Amersham) following the manufacturer's protocol.

The packaged DNA library was amplified on solid medium. This was accomplished by incubating 0.1 ml *E. coli* strain NM539 plating cells suspended in 10 mM  $\text{MgSO}_4$  with 15 - 25  $\mu$ L of the packaged DNA library at 37°C for

15 minutes. Bacteria with adsorbed phage were plated onto BBL plates (10 g BBL trypticase peptone, 5 g NaCl and 15 g agar per litre) using 3 mL of BBL top-agarose (same as BBL plates except agar replaced with 0.6% agarose) and plates were incubated overnight at 37°C. Phage were eluted from the top-agarose by adding 3 mL SM buffer (50 mM Tris-HCl, pH 7.5, 8 mM MgSO<sub>4</sub>, 100 mM NaCl, 0.01% gelatin) to the plates and leaving them at 4°C for 7 hours. SM buffer containing phage was collected from the plates, transferred to a screwcap tube and stored at 4°C over chloroform.

#### Example 10

This Example describes the cloning of a gene encoding the *M. catarrhalis* 200 kDa outer membrane protein.

The *M. catarrhalis* genomic library in phage lambda EMBL3 was screened using an anti-200 kDa protein guinea pig antiserum. A lambda phage clone 8II, which expressed an about 200 kDa protein, was confirmed by immunoblotting of the phage lysate using the about 200 kDa outer membrane-specific antiserum.

Plate lysate cultures of this recombinant phage were prepared. The DNA was extracted from the plate lysates using a Wizard Lambda Preps DNA Purification System (Promega Corp, Madison, WI) according to the manufacturer's instructions. This phage clone carried a DNA insert of about 16 kb in size (the restriction map for which is shown in Figure 5). The phage DNA was digested with a mixture of the restriction enzymes SalI and XhoI, and separated by agarose gel electrophoresis. Two DNA bands, approximately 5 kb and 11 kb in size, respectively, were cut out from the gel and extracted using a GeneClean kit (BIO 101 Inc., LaJolla, CA) according to the manufacturer's direction.

The smaller 5 kb fragment was ligated into a plasmid vector, pBluescript II SK +/- (Stratagene Cloning



Systems, LaJolla, CA), which had been previously digested with SalI and XhoI, to produce plasmid pKS5. The larger 11 kb fragment was ligated into a plasmid vector, pSP72 (Promega Corp., Madison, WI), to produce plasmid pKS9.

5 Both ligated plasmids were used to transform *E. coli*, strain DH5 $\alpha$ .

The lambda phage DNA was also digested with a mixture of XhoI and KpnI and the approximately 1.2 kb fragment was isolated after agarose gel separation as  
10 described above. This 1.2 kb fragment was ligated into a plasmid vector, pGEM-7Zf(+) (Promega Corp., Madison, WI), to produce plasmid pKS47. Restriction maps of the plasmid clones are shown in Figure 5.

#### Example 11

15 This Example describes the sequencing of the gene encoding the about 200 kDa outer membrane protein of *M. catarrhalis*.

The gene encoding the about 200 kDa outer membrane protein was sequenced using an Applied Biosystems  
20 sequencer. The one strand of the insert in the plasmid pKS5, was sequenced after construction of a nested set of deletions using a Erase-a-Base system (Promega Corp., Madison, WI). The plasmid pKS5 was first digested with XhoI and KpnI, treated with exonuclease III to generate  
25 a nested set of deletions in the insert and then recircularized according to the manufacturer's directions. *E. coli* DH5 $\alpha$  was transformed with a series of plasmids with deletions generated in this way. Plasmids were isolated from the transformants using a  
30 Quiagen midi plasmid isolation kit (Qiagen) and the size of plasmids examined by agarose gel electrophoresis after restriction enzyme digestion. The inserts of the plasmids with deletions were sequenced using a bacteriophage T7 promoter sequence as a primer.

35 Based upon the sequence, nucleotide primers were synthesized. Using the synthetic nucleotide primers,

sequence gaps, which were not sequenced by the Erase-a Base system, were determined.

The sequences of the inserts in plasmids pKS47 and pKS71 were determined from both ends using synthetic nucleotide primers. The nucleotide sequence of the gene has an open reading frame of the gene coding for the about 200 kDa outer membrane protein of *M. catarrhalis* as shown in Figure 6 (SEQ ID No: 2). This sequence included a nucleotide sequence:

10        5'- AATGTCAAATCAGTCATTAACAAAGAACAAGTAAATGATGCCAATA  
          AAAAGCAAGGCATC-3' (SEQ ID No: 9)

which encodes the internal amino acid sequence of the about 200 kDa outer membrane protein (SEQ ID No: 5) determined above. This result confirms that the cloned gene has an open reading frame of the gene coding for the about 200 kDa outer membrane protein of *M. catarrhalis*. The gene encodes a protein having 1992 amino acids, a calculated molecular weight of 204,677 and a calculated amino acid composition as shown in Table III below. The deduced amino acid sequence of the protein is shown in Figure 6 (SEQ ID No: 3).

#### Example 12

This Example describes the identification of the start codon of the gene encoding the about 200 kDa gene of *M. catarrhalis*.

To identify the translation start codon and the promoter region of the 200 kDa protein gene, a plasmid, pKS80, was constructed from pKS5 and pKS47 (Fig. 5). This construct contained about 250 base pairs of DNA upstream from the ATG. The plasmid, pKS5, was digested with KpnI and XhoI. The digest was separated on 0.8% agarose gel and the about 8 kb DNA fragment was cut out from the gel and extracted. Another plasmid, pKS47, was also digested with the two enzymes and the about 1.1 kb DNA fragment was extracted. The 1.1 kb fragment was ligated to the 8 kb fragment to construct pKS80. Western

blots using anti-200 kD protein guinea pig serum failed to detect 200 kD protein in the lysates of the transformants carrying pKS80.

To examine if the construct was too long to be expressed in *E. coli*, three different sizes of C-terminal truncations were constructed, as shown in Fig. 8. First, the whole insert in pKS80 was cut out by digestion with KpnI and BamHI and then inserted into another vector plasmid, pGEM7Zf(+) (Promega, Madison WI), which had been previously digested with the same two enzymes. The resulting plasmid, pKS105, was further digested with either one of the following enzymes, (1) HindIII, (2) HpaI and SmaI or (3) EcoRV, gel-purified and then recircularized to produce pKS130, pKS136 and pKS144, respectively. Transformants of *E. coli*, DH5 $\alpha$ , with either one of pKS130, pKS136 or pKS144 did not produce any truncated proteins, when examined on Western blots using anti-200 kD protein guinea pig serum.

Next, to investigate if the start codon was GTG and if the promoter region was further upstream from the GTG, an about 0.9 kb fragment was cut out from pKS71 using ApaI and KpnI, and ligated into pKS130, pKS136 and pKS144, which had been previously digested with ApaI and KpnI. The 0.9kb fragment from pKS71 carried the NcoI-KpnI fragment, which contained the possible start codon, GTG, and about 700 bp upstream region from the GTG (Fig. 8). The resulting constructs, pKS159, pKS149 and pKS155, produced truncated proteins, which were recognized by anti-200 kDa protein guinea pig serum on Western blots. The ApaI and KpnI fragment was also ligated to pKS105, which had no C-terminal truncation, to produce pKS164. The transformants carrying pKS164 produced a full-length 200 kDa protein, which was recognized by the same antiserum on Western blot. These results show that the 5'- region of the gene containing the GTG codon and its upstream sequence is necessary for expression of the

about 200 kDa protein gene from its own promoter in *E. coli*, and indicate that a translation start codon of the about 200 kDa protein gene is GTG.

To confirm that the start codon of the gene is GTG,  
5 two peptides were synthesized, as shown in Fig. 9, according to the deduced amino acid sequence from the nucleotide sequence in Fig. 6. Peptide 1 (SEQ ID No: 12) encompasses the 30 amino acids from the GTG start codon. Peptide 2 (SEQ ID No: 12) is the next 30 amino acid  
10 peptide. The peptides are identified in Figure 6 by underlining. Antisera were raised against these two peptides in guinea pigs and antisera were obtained. As seen in Fig. 10, antisera raised against these two peptides clearly recognized 200 kDa protein from *M.*  
15 *catarrhalis*, strain 4223, by Western blotting. *M. catarrhalis*, strain 4223, was sonicated. Proteins in the sonicate were separated on a SDS-PAGE gel and transferred to PVDF membrane. The membrane was cut into strips and treated with either anti-peptide 1 or anti-peptide 2  
20 guinea pig serum as a first antibody. The second antibody was goat anti-guinea pig IgG conjugated with horse radish peroxidase (Jackson ImmunoResearch Lab. Inc., West Grove, PA). The membrane was finally treated with CN/DAB substrate (Pierce, Rockford, IL) for color  
25 development. Lane 1: prestained molecular weight marker, Lane 2: anti-200 kD protein serum, Lane 3: anti-peptide I serum from guinea pig No. 1, Lane 4: prebleed serum from guinea pig No. 1, Lane 5: anti-peptide 1 serum from guinea pig No. 2, Lane 6: prebleed serum from guinea pig  
30 No. 2, Lane 7: anti-peptide 2 serum from guinea pig No. 3, Lane 8: prebleed serum from guinea pig No. 3, Lane 9: anti-peptide 2 serum from guinea pig No. 4, Lane 10: prebleed serum from guinea pig No. 4. The results shown in Figure 10 indicate that the GTG is the translation  
35 start codon of the gene encoding the about 200 kDa protein.

5 gene is shown in Fig. 5. The sequence between NcoI and  
SalI and its amino acid translation are shown in Fig. 6.  
The calculated amino acid composition of the about 200  
kDa protein is shown in Table III.

10 truncation genes under the control of the T7 promoter (as shown in Fig. 11), a ScaI-SalI fragment, which carried the about 1.9kb 3'- region of the about 200 kDa protein gene, was cut out from pKS5, and the PvuII-SalI fragment, which carried the about 4.8 kb 3'- region, was cut out  
15 from pKS80. The two fragments were ligated into a plasmid, pT7-7, previously digested with SmaI and SalI, to produce pKS94 and pKS91, respectively. These ligations resulted in fusions of 1.9 kb and 4.8 kb 3'- regions with seven N-terminal amino acids from the  
20 vector. When transformants of an *E. coli* strain, BL21(DE3)/pLyss, with either pKS94 or pKS91 were induced with IPTG, they produced a large quantity of N-terminally truncated 200 kDa protein. Fig. 12 shows a Western blot showing the expression of the truncated protein by one of  
25 transformants carrying the pKS94 plasmid.

30 digestion with PstI, gel-purified, and then ligated to  
pGEM5Zf(+) (Promega, Madison, WI), previously digested  
with the same enzyme. The *E. coli* DH5 $\alpha$  clones, which  
carried the gene in the same direction and reading frame  
as the LacZ  $\alpha$  peptide, were selected by restriction  
35 enzyme analyses. These clones constitutively expressed  
the fusion protein, as shown in Fig. 13.

SUMMARY OF THE DISCLOSURE

In summary of the disclosure, the present invention provides an isolated and purified outer membrane protein of a *Moraxella* strain, particularly *M. catarrhalis*,  
5 having a molecular weight of about 200 kDa as well as isolated and purified DNA molecules encoding the outer membrane protein. The invention also provides analogs, truncations and peptides corresponding to portions of the outer membrane protein. The protein, DNA sequences,  
10 recombinant proteins derived therefrom and peptides are useful for diagnosis, immunization and the generation of diagnostic and immunological reagents. Modifications are possible within the scope of this invention.

**TABLE I**

Presence of the about 200 kDa outer membrane protein in various  
isolates of *Moraxella catarrhalis*

---

<u>Type of Clinical Isolate</u>	<u>Number of isolates Examined</u>	<u>Number of isolates <sup>1</sup> containing the 200 kDa outer membrane protein</u>
Otitis Media	37	36
Sputum/Expectoration/Bronchial Secretion	13	6
Blood	2	2
Nasopharynx	1	1
Unknown	1	0

<sup>1</sup> The presence of the about 200 kDa outer membrane protein was determined by immunoblot analysis using a monospecific guinea pig anti-200 kDa protein antiserum.

**TABLE II**

Detection of about 200kDa outer membrane protein of *M. catarrhalis* by the monospecific anti-200kDa outer membrane guinea pig antiserum

---

<u>Strain</u>	<u>Sample</u>	<u>Reciprocal Reactive Titre</u>
4223	Whole cells not fixed	800
RH408	Whole cells not fixed	<200
H12	Whole cells not fixed	<200
<i>E. coli</i> BL21	Whole cells not fixed	<200

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4223	Whole cells fixed	3200
RH408	Whole cells fixed	200
H12	Whole cells fixed	<200
<i>E. coli</i> BL21	Whole cells fixed	<200

4223	Sonicate	12,800
RH408	Sonicate	800
H12	Sonicate	800
<i>E. coli</i> BL21	Sonicate	200



**TABLE III**

Amino acid composition of the about 200 kDa outer  
membrane protein of *M. catarrhalis*

---

<u>Residue</u>	<u>Number</u>	<u>Percentage (MW)</u>
N - Asparagine	196	10.9
T - Threonine	221	10.9
K - Lysine	159	10.0
D - Aspartic Acid	147	8.3
A - Alanine	219	7.6
V - Valine	148	7.2
I - Isoleucine	116	6.4
S - Serine	150	6.4
G - Glycine	222	6.2
L - Leucine	111	6.1
Q - Glutamine	83	5.2
E - Glutamic Acid	55	3.5
F - Phenylalanine	40	2.9
R - Arginine	34	2.6
Y - Tyrosine	27	2.2
H - Histidine	24	1.6
P - Proline	30	1.4
M - Methionine	7	.4
W - Tryptophan	3	.3
B - Aspartic Acid Asparagine	0	.0
C - Cysteine	0	.0

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CLAIMS

What we claim is:

1. An isolated and purified outer membrane protein of a *Moraxella* strain having an apparent molecular mass of about 200 kDa, as determined by SDS-PAGE or a fragment or an analog thereof.
2. The protein of claim 1 wherein the *Moraxella* strain is *Moraxella catarrhalis*.
3. The protein of claim 2 wherein the strain is *Moraxella catarrhalis* 4223.
4. The protein of claim 1 containing the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-Lys-Gln-Gly-Ile (SEQ ID No: 10) for *Moraxella catarrhalis* strain 4223 or containing the corresponding amino acid sequence from other *Moraxella* strains.
5. The protein of claim 1 which is at least about 70 wt% pure.
6. The protein of claim 5 which is at least about 95 wt% pure.
7. The protein of claim 1 in the form of an aqueous solution thereof.
8. The protein of claim 1 recognizable by an antibody preparation specific for a peptide having the amino acid sequence of NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-Lys-Gln-Gly-Ile (SEQ ID No: 10).
9. The protein of claim 1 having substantially the amino acid composition as shown in Table III.
10. A purified and isolated nucleic acid molecule encoding an outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, as determined by SDS-PAGE, or a fragment or an analog of the outer membrane protein.
11. The nucleic acid molecule of claim 10, wherein the strain of *Moraxella* is a strain of *Moraxella catarrhalis*.

12. The nucleic acid molecule of claim 11, wherein the strain is *Moraxella catarrhalis* 4223.

13. The nucleic acid molecule of claim 10, wherein the encoded protein contains the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-Lys-Gln-Gly-Ile (SEQ ID No: 10) for *Moraxella catarrhalis* strain 4223 or contains the corresponding amino acid sequence from other *Moraxella* strains.

14. A purified and isolated nucleic acid molecule having a sequence selected from the group consisting of:

(a) a DNA sequence as set out in Figure 6 (SEQ ID Nos: 1 and 2), or the complementary sequence thereto;

(b) a DNA sequence encoding a 200-kDa protein of a strain of *Moraxella* and containing the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-Lys-Gln-Gly-Ile (SEQ ID No: 10), or the complementary sequence thereto;

(c) a DNA sequence encoding a deduced amino acid sequence as set out in Figure 6 (SEQ ID No: 3), or the complimentary sequence thereto; and

(d) a nucleotide sequence which hybridizes under stringent conditions to any one of the sequences defined in (a), (b) or (c).

15. The nucleic acid molecule of claim 14, wherein the nucleotide sequence defined in (d) has at least about 90% sequence identity with any one of the sequences defined in (a), (b) or (c).

16. A vector adapted for transformation of a host comprising the nucleic acid molecule of claim 10 or 14.

17. An expression vector adapted for transformation of a host comprising the nucleic acid molecule of claim 10 or 14 and expression means operatively coupled to the nucleic acid molecule for expression by the host of said outer membrane protein of a strain of *Moraxella* or the fragment or the analog of the outer membrane protein.

18. The expression vector of claim 17, wherein the expression means includes a nucleic acid portion encoding a leader sequence for secretion from the host of the outer membrane protein or the fragment or the analog of the outer membrane protein.

19. The expression vector of claim 17, wherein the expression means includes a nucleic acid portion encoding a lipidation signal for expression from the host of a lipidated form of the outer membrane protein or the fragment or the analog of the outer membrane protein.

20. A transformed host containing an expression vector as claimed in claim 17.

21. A recombinant outer membrane protein or fragment or analog thereof producible by the transformed host of claim 20.

22. A live vector for delivery of an outer membrane protein of a strain of *Moraxella* having a molecular weight of about 200 kDa or a fragment or analog thereof to a host, comprising a vector containing the nucleic acid molecule of claim 10 or 14.

23. The live vector of claim 21, wherein the vector is selected from the group consisting of *E. coli*, *Salmonella*, *Mycobacteria*, adenovirus, poxvirus, vaccinia and poliovirus.

24. A peptide having no less than six amino acids and no more than 150 amino acids and containing an amino acid sequence corresponding to a portion only of an outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, as determined by SDS-PAGE, or a fragment or of an analog of the outer membrane protein.

25. The peptide of claim 24, wherein the *Moraxella* strain is a *Moraxella catarrhalis* strain.

26. The peptide of claim 25, wherein the strain is *Moraxella catarrhalis* 4223.

27. The peptide of claim 24 having the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-x-Gln-Gly-Ile (SEQ ID No: 5) or NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys (SEQ ID No: 6) for the *Moraxella catarrhalis* 4223 strain or the amino acid sequence for the corresponding peptide for other strains of *Moraxella*.

28. An immunogenic composition, comprising at least one active component selected from the group consisting of:

(A) an isolated and purified outer membrane protein of a *Moraxella* strain having a molecular mass of about 200 kDa, as determined by SDS-PAGE, or a fragment or an analog thereof;

(B) a purified and isolated nucleic acid molecule encoding an outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, as determined by SDS-PAGE or a fragment or an analog thereof;

(C) a purified and isolated nucleic acid molecule having a sequence selected from the group consisting of:

(a) a DNA sequence set out in Figure 6 (SEQ ID No: 1 or 2), or the complementary sequence thereto;

(b) a DNA sequence encoding an about 200 kDa protein of a strain of *Moraxella* and containing the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-Lys-Gln-Gly-Ile (SEQ ID No: 10), or the complementary sequence thereto;

(c) a DNA sequence encoding an amino acid sequence as set forth in Figure 6 (SEQ ID No: 3), or the complementary sequence thereto; and

(d) a nucleotide sequence which hybridizes under stringent conditions to any one of the sequences defined in (a), (b) or (c);

(D) a recombinant outer membrane protein or fragment or analog thereof producible in a transformed



host containing an expression vector comprising a nucleic acid molecule as defined in (B) or (C) and expression means operatively coupled to the nucleic acid molecule for expression by the host of the recombinant outer membrane protein or fragment or analog thereof;

(E) a live vector, comprising a vector containing a purified and isolated nucleic acid molecule encoding a protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, as determined by SDS-PAGE, or a fragment or analog thereof;

(F) a live vector, comprising a vector containing a purified and isolated nucleic acid molecule having a sequence selected from the group consisting of:

(a) a DNA sequence as set out in Figure 6 (SEQ ID No: 1), or the complementary sequence thereto;

(b) a DNA sequence encoding an about 200 kDa protein of a strain of *Moraxella* and containing the amino acid sequence NH<sub>2</sub>-Asn-Val-Lys-Ser-Val-Ile-Asn-Lys-Glu-Gln-Val-Asn-Asp-Ala-Asn-Lys-Lys-Gln-Gly-Ile (SEQ ID No: 10), or the complementary sequence thereto;

(c) a DNA sequence encoding an amino acid sequence as set forth in Figure 6 (SEQ ID No: 3), or the complimentary sequence thereto; and

(d) a nucleotide sequence which hybridizes under stringent conditions to any one of the sequences defined in (a), (b) or (c); and

(G) a peptide having no less than six amino acids and no more than 150 amino acids and containing an amino acid sequence corresponding to a portion only of an outer membrane protein of a strain of *Moraxella* having a molecular mass of about 200 kDa, as determined by SDS-PAGE, or of an analog of the outer membrane protein; and

a pharmaceutically acceptable carrier therefor, said at least one active component producing an immune response when administered to a host.

29. The immunogenic composition of claim 28 formulated as a vaccine for *in vivo* administration to a host to confer protection against disease caused by a strain of *Moraxella*.

30. The immunogenic composition of claim 29 wherein the strain is *Moraxella catarrhalis*.

31. The immunogenic composition of claim 29 formulated as a microparticle, capsule, ISCOM, or liposome preparation.

32. The immunogenic composition of claim 29 in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces.

33. The immunogenic composition of claim 29 further comprising at least one other immunogenic or immunostimulating material.

34. The immunogenic composition of claim 33 wherein the at least one other immunostimulating material is at least one adjuvant.

35. The immunogenic composition of claim 34 wherein the at least one adjuvant is selected from the group consisting of aluminum phosphate, aluminum hydroxide, QS21, Quil A, derivatives and components thereof, ISCOM matrix, calcium phosphate, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octodecyl ester of an amino acid, a muramyl dipeptide, polyphosphazene, ISCOPREP, DC-chol, DDBA and a lipoprotein.

36. The immunogenic composition of claim 35 wherein the host is a primate.

37. The immunogenic composition of claim 36 wherein the primate is a human.

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Figure 1A

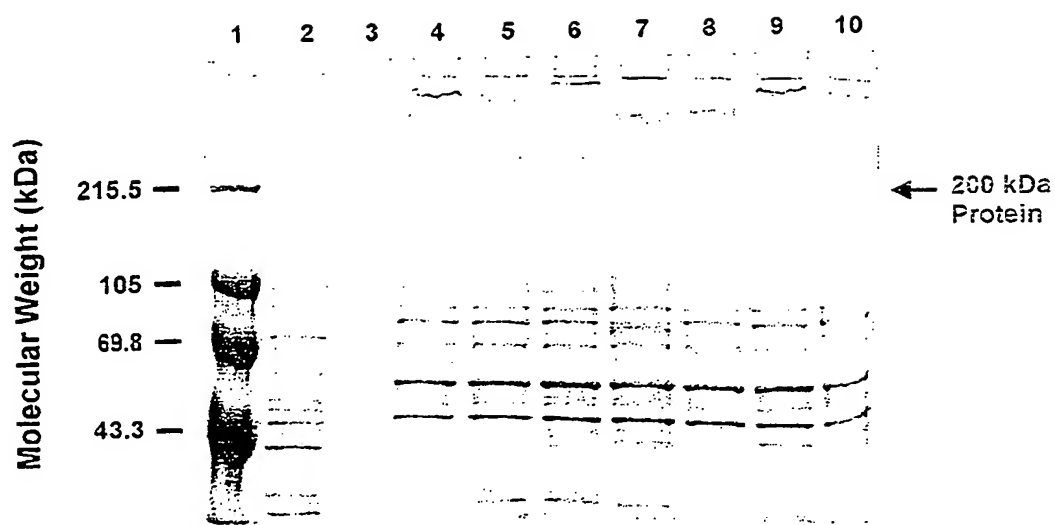
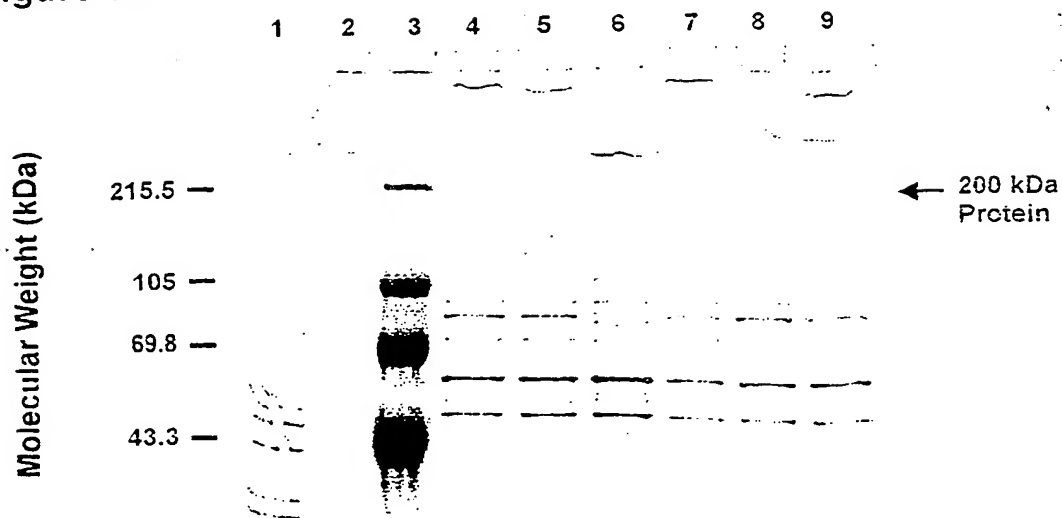


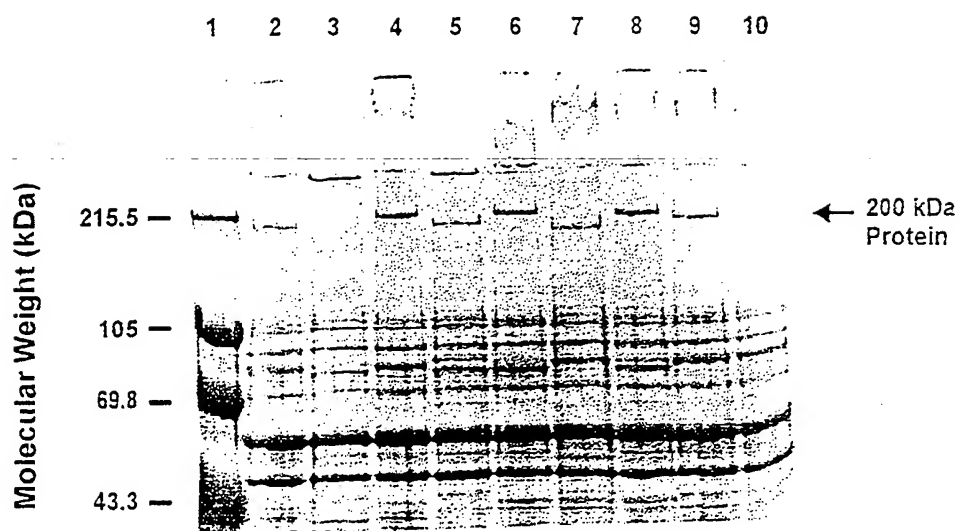
Figure 1B



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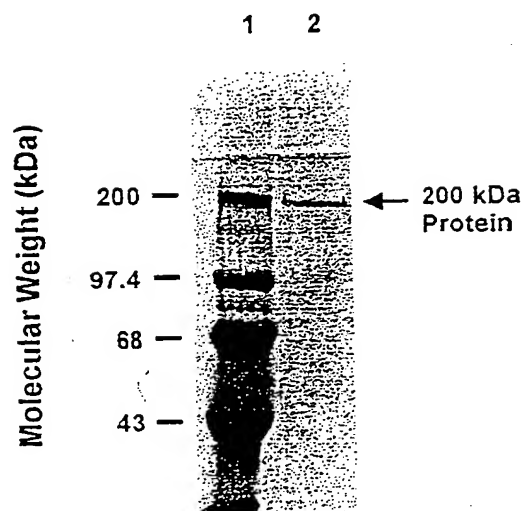
Figure 2



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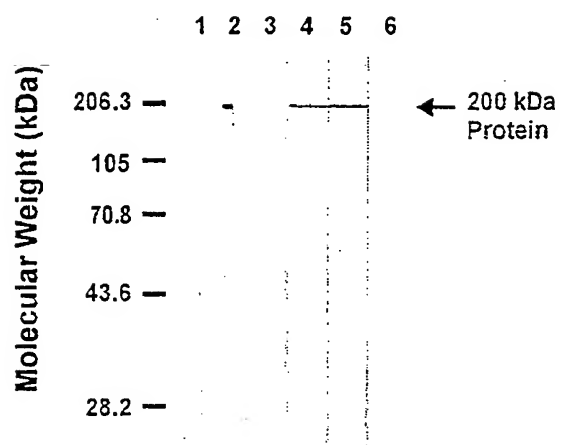
Figure 3



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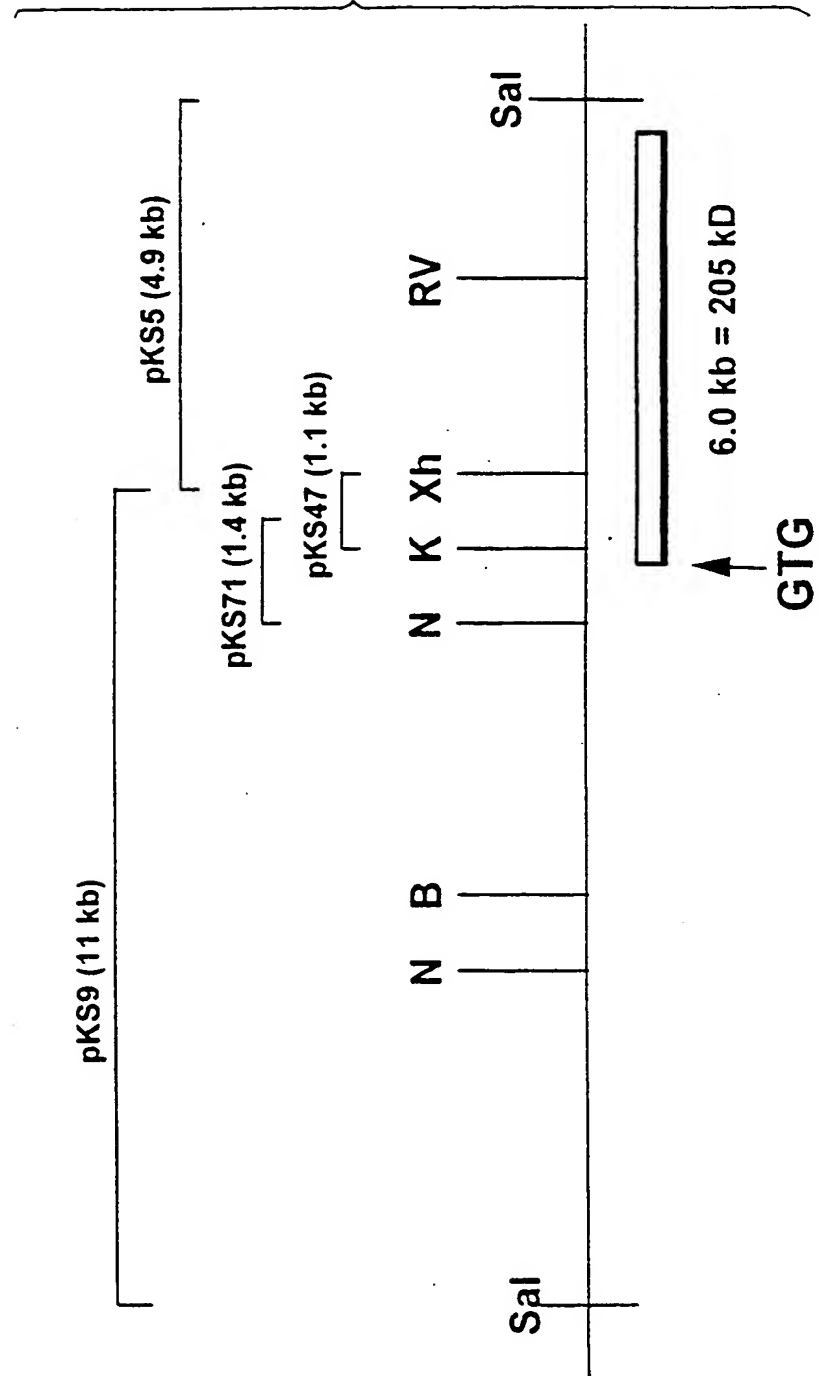
Figure 4



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FIG.5.



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## FIG.6.

CC	ATG	GAT	ATG	GGC	AGG	TGT	GCT	CGC	CTG	CCG	TAT	GAT	GGC	GAT	GAC	ACC	CCA	TTT	54
																			27
GCC	CCA	TAT	CTG	TAC	GAT	TTG	ACA	TGT	GAT	ATG	ATT	TAA	CAT	GTG	ACA	TGA	TTT	108	
																			81
AAC	ATT	GTT	TAA	TAC	TGT	TGC	CAT	CAT	TAC	CAT	AAT	TTA	GTA	ACG	CAT	TTA	GTA	162	
																			135
ACG	CAT	TTG	TAA	AAA	TCA	TTG	CGC	CCC	TTT	ATG	TGT	ATC	ATA	TGA	ATA	GAA	TAT	216	
																			189
TAT	GAT	TGT	ATC	TGA	TTA	TTG	TAT	CAG	AAT	GGT	GAT	GCT	ATA	TGA	TGA	TGC	CTA	270	
																			243

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FIG.6 con't.

CGA GTT GAT TTG GGT TAA TCA CTC TAT GAT TTG ATA TAT TTT GAA ACT AAT CTA  
297 324

TTG ACT TAA ATC ACC ATA TGG TTA TAA TTT AGC ATA ATG GTA GGC TTT TTG TAA  
351 378

AAA TCA CAT CGC AAT ATT GTT CTA CTG TTA CTA CCA TGC TTG AAT GAC GAT CCC  
405 432

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## FIG.6 con't.

486  
 AAT CAC CAG ATT CAT TCA AGT GAT GTG TTT GTA TAC GCA CCA TTT ACC CTA ATT

513  
 ATT TCA ATC AAA TGC CTA TGT CAG CAT GTA TCA TTT TTT TAA GGT AAA CCA CCA

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540  
 567  
 TGA ATC ACA TCT ATA AAG TCA TCT TTA ACA AAG CCA CAG GCA CAT TTA TGG CAG

594  
 621  
 TGG CAG AGT ACG CCA AAT CCC ACA GCA CGG GGG GGG GGT AGC TGT GCT ACA GGG

648

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## FIG.6 con't.

CAA GTT GGC AGT GTA TGC ACT CTG AGC TTT GCC CGT ATT GCC GCG CTC GCT GTC 702

CTC GTG ATC GGT GCA ACG CTC AGT GGC AGT GCT TAT GCT CAA AAA AAA GAT ACC 756  
Met Ile Gly Ala Thr Leu Ser Gly Ser Ala Tyr Ala Gln Lys Lys Asp Thr

## Peptide 1

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AAA CAT ATC GCA ATT GGT GAA CAA AAC CAG CCA AGA CGC TCA GGC ACT GCC AAG 810  
Lys His Ile Ala Ile Gly Glu Gln Asn Gln Pro Arg Arg Ser Gly Thr Ala Lys

GCG GAC GGT GAT CGA GCC ATT GCT ATT GGT GAA AAT GCT AAC GCA CAG GGC GGT 864  
Ala Asp Gly Asp Arg Ala Ile Ala Ile Gly Glu Asn Ala Asn Ala Gln Gly Gly

## Peptide 2

CAA GCC ATC GCC ATC GGT AGT AGT AAT AAA ACT GTC AAT GGA AGC AGT TTG GAT 918  
Gln Ala Ile Ala Ile Gly Ser Ser Asn Lys Thr Val Asn Gly Ser Ser Leu Asp

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## FIG.6 con't.

AAG ATA GGT ACC GAT GCT ACG GGT CAA GAG TCC ATC GCC ATC GGT GGT GAT GTA	945	972
Lys Ile Gly Thr Asp Ala Thr Gly Gln Glu Ser Ile Ala Ile Gly Gly Asp Val		
AAG GCT AGT GGT GAT GAT GCC TCG ATT GCC ATC GGT AGT GAT GAC TTA CAT TTG CTT	999	1026
Lys Ala Ser Gly Asp Ala Ser Ile Ala Ile Ile Gly Ser Asp Asp Leu His Leu Leu		
GAT CAG CAT GGT AAT CCT AAA CAT CCG AAA GGT ACT CTG ATT AAC GAT CTT ATT	1053	1080
Asp Gln His Gly Asn Pro Lys His Pro Lys Gly Thr Leu Ile Asn Asp Leu Ile		
AAC GGC CAT GCA GTA TTA AAA GAA ATA CGA AGC TCA AAG GAT AAT GAT GTA AAA	1107	1134
Asn Gly His Ala Val Leu Lys Glu Ile Arg Ser Ser Lys Asp Asn Asp Val Lys		

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## FIG.6 con't.

1161  
 TAT AGA CGC ACA ACC GCA AGC GGA CAC GCC AGT ACT GCA GTG GGA GCC **ATG** TCA  
 Tyr Arg Arg Thr Thr Ala Ser Gly His Ala Ser Thr Ala Val Gly Ala **MET** Ser

1188

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1215  
 TAT GCA CAG GGT CAT TTT TCC AAC GCC TTT GGT ACA CGG GCA ACA GCT AAA AGT  
 Tyr Ala Gln Gly His Phe Ser Ser Asn Ala Phe Gly Thr Arg Ala Thr Ala Lys Ser

1242

1269  
 GCC TAT TCC TTG GCA GTG GGT CTT GCC GCC ACA GCC GAG GGC CAA TCT ACA ATC  
 Ala Tyr Ser Leu Ala Val Gly Leu Ala Ala Thr Ala Glu Gly Gln Ser Thr Ile

1296

1323  
 GCT ATT GGT TCT GAT GCA ACA TCT AGC TCG TTG GGA GCG ATA GCC CTT GGT GCA  
 Ala Ile Gly Ser Asp Ala Thr Ser Ser Ser Leu Gly Ala Ile Ala Leu Gly Ala

1350

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## FIG.6 con't.

1377  
GGT ACT CGT GCT CAG CTA CAG GGC AGT ATT GCC CTA GGT CAA GGT TCT GTT GTC  
Gly Thr Arg Ala Gln Leu Gln Gly Ser Ile Ala Leu Gly Gln Gly Ser Val Val

1431  
ACT CAG AGT GAT AAT AAT TCT AGA CCG GCC TAT ACA CCA AAT ACC CAG GCA CTA  
Thr Gln Ser Asp Asn Asn Ser Arg Pro Ala Tyr Thr Pro Asn Thr Gln Ala Leu

1485  
GAC CCC AAG TTT CAA GCC ACC AAT AAT ACG AAG GCG GGT CCA CTT TCC ATT GGT  
Asp Pro Lys Phe Gln Ala Thr Asn Asn Thr Lys Ala Gly Pro Leu Ser Ile Gly

1539  
AGT AAC TCT ATC AAA CGT AAA ATC ATC AAT GTC GGT GCA GGT GGT AAT AAA ACC  
Ser Asn Ser Ile Lys Arg Lys Ile Ile Asn Val Gly Ala Gly Val Asn Lys Thr

1593  
GAT GCG GTC AAT GTG GCA CAG CTA GAA GCG GTG GTG AAG TGG GCT AAG GAG CGT  
Asp Ala Val Asn Val Ala Gln Leu Glu Ala Val Val Lys Trp Ala Lys Glu Arg

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## FIG.6 con't.

AGA	ATT	ACT	TTT	CAG	GGT	GAT	GAT	AAC	AGT	ACT	GAC	GTA	AAA	ATA	GGT	TTG	GAT	1674
Arg	Ile	Thr	Phe	Gln	Gly	Asp	Asp	Asn	Ser	Thr	Asp	Val	Lys	Ile	Gly	Leu	Asp	
																		1647
AAT	ACT	TTA	ACT	ATT	AAA	GGT	GGT	GCA	GAG	ACC	AAC	GCA	TTA	ACC	GAT	AAT	AAT	1728
Asn	Thr	Leu	Thr	Ile	Lys	Gly	Gly	Ala	Glu	Thr	Asn	Ala	Leu	Thr	Asp	Asn	Asn	1701
																		1728
ATC	GGT	GTG	GTA	AAA	GAG	GCT	GAT	AAT	AGT	GGT	CTG	AAA	GTT	AAA	CTT	GCT	AAA	1782
Ile	Gly	Val	Val	Lys	Glu	Ala	Asp	Asn	Ser	Gly	Leu	Lys	Val	Lys	Leu	Ala	Lys	
																		1755
ACT	TTA	AAC	AAT	CTT	ACT	GAG	GTG	AAT	ACA	ACT	ACA	TTA	AAT	GCC	ACA	ACC	ACA	1836
Thr	Leu	Asn	Asn	Leu	Thr	Glu	Val	Asn	Thr	Thr	Thr	Leu	Asn	Ala	Thr	Thr	Thr	1809

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[illegible]

BNSDOCID: <WO 9634960A1 J>



## FIG.6 con't.

2079  
 AAA CAA GCA CCA TAT TTG GAT AAA AAA CAA CTT AAA GTG GGT AGT GTT GCA ATT  
 Lys Gln Ala Pro Tyr Leu Asp Lys Lys Gln Leu Lys Val Gly Ser Val Ala Ile

2106

2133  
 ACC ATA GAC AAT GGC ATT GAT GAT GCA GGT AAT AAA AAG ATC AGT AAT CTT GCC AAA  
 Thr Ile Asp Asn Gly Ile Asp Ala Gly Asn Lys Lys Ile Ser Asn Leu Ala Lys

2160

2187  
 GGT AGC AGT GCT AAC GAT GCG GTT ACC ATC GAA CAG CTC AAA GCC GCC AAG CCT  
 Gly Ser Ser Ala Asn Asp Ala Val Thr Ile Glu Gln Leu Lys Ala Ala Lys Pro

2214

2241  
 ACT TTA AAC GCA GGC GCT GGC ATC AGT GTC ACA CCT ACT GAA ATA TCA GTT GAT  
 Thr Leu Asn Ala Gly Ala Gly Ile Ser Val Thr Pro Thr Glu Ile Ser Val Asp

2268

2295  
 GCT AAG AGT GGC AAT GTT ACC GCC CCA ACT TAC AAC ATT GGC GTG AAA ACC ACC  
 Ala Lys Ser Gly Asn Val Thr Ala Pro Thr Tyr Asn Ile Gly Val Lys Thr Thr

2322

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## FIG.6 con't.

GAG CTT AAC AGT GAT GGC ACT AGT GAT AAA TTT AGT GTT AAG GGT AGT GGT ACG	2349	2376
Glu Leu Asn Ser Asp Gly Thr Ser Asp Lys Phe Ser Val Lys Gly Ser Gly Thr		
AAC AAT AGC TTA GTT ACC GCC GAA CAT TTG GCA AGC TAT CTA AAT GAA GTC AAT	2403	2430
Asn Asn Ser Leu Val Thr Ala Glu His Leu Ala Ser Tyr Leu Asn Glu Val Asn		16/47
CGA ACG GCT GAC AGT GCT CTA CAA AGC TTT ACC GTT AAA GAA GAA GAC GAT GAT	2457	2484
Arg Thr Ala Asp Ser Ala Leu Gln Ser Phe Thr Val Lys Glu Glu Asp Asp Asp		
GAC GCC AAC GCT ATC ACC GTG GCT AAA GAT ACG ACA AAA AAT GCC GGC GCA GTC	2511	2538
Asp Ala Asn Ala Ile Thr Val Ala Lys Asp Thr Thr Lys Asn Ala Gly Ala Val		

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## FIG.6 con't.

2592  
 AGC ATC TTA AAA CTC AAA GGT AAA AAC GGT CTA ACG GTT GCT ACC AAA AAA GAT  
 Ser Ile Leu Lys Lys Leu Lys Gly Lys Asn Gly Leu Thr Val Ala Thr Lys Lys Asp

2646  
 GGT ACG GTT ACC TTT GGG CTT AGC CAA GAT AGC GGT CTG ACC ATT GGC AAA AGC  
 Gly Thr Val Thr Phe Gly Leu Ser Gln Asp Ser Gly Leu Thr Ile Gly Lys Ser

2700  
 ACC CTA AAC AAC GAT GGC TTG ACT GTT AAA GAT ACC AAC GAA CAA ATC CAA GTC  
 Thr Leu Asn Asn Asp Gly Leu Thr Val Lys Asp Thr Thr Asn Glu Gln Ile Gln Val

2754  
 GGT GCT AAT GGC ATT AAA TTT ACT AAT GTG AAT AAT CCA GGT ACT GGC  
 Gly Ala Asn Gly Ile Lys Phe Thr Asn Val Asn Gly Ser Asn Pro Gly Thr Gly

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## FIG.6 con't.

ATT GCA AAT ACC GCT CGC ATT ACC AGA GAT AAA ATT GGC TTT GCT GGT TCT GAT	2781	2808
Ile Ala Asn Thr Ala Arg Ile Thr Arg Asp Lys Ile Gly Phe Ala Gly Ser Asp		
GGT GCA GTT GAT ACA AAC AAA CCT TAT CTT GAT CAA GAC AAG CTA CAA GTT GGC	2835	2862
Gly Ala Val Asp Thr Asn Lys Pro Tyr Leu Asp Gln Asp Lys Leu Gln Val Gly		
AAT GTT AAG ATT ACC AAC ACT GGC ATT AAC GCA GGT GGT AAA GCC ATC ACA GGG	2889	2916
Asn Val Lys Ile Thr Asn Thr Gly Ile Asn Ala Gly Gly Lys Ala Ile Thr Gly		
CTG TCC CCA ACA CTG CCT AGC ATT GCC GAT CAA AGT AGC CGC AAC ATA GAA CTG	2943	2970
Leu Ser Pro Thr Leu Pro Ser Ile Ala Asp Gln Ser Ser Arg Asn Ile Glu Leu		
GGC AAT ACA ATC CAA GAC AAA GAC AAA TCC AAC GCT GCC AGC ATT AAT GAT ATA	2997	3024
Gly Asn Thr Ile Gln Asp Lys Asp Lys Ser Ser Asn Ala Ala Ser Ile Asn Asp Ile		

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## FIG.6 con't.

3051 TTA AAT ACA GGC TTT AAC CTA AAA AAT AAT AAC AAC CCC ATT GAC TTT GTC TCC  
 Leu Asn Thr Gly Phe Asn Leu Lys Asn Asn Asn Asn Asn Pro Ile Asp Phe Val Ser 3078

3105 ACT TAT GAC ATT GTT GAC TTT GCC AAT GGC AAT GCC ACC ACC GCC ACA GTA ACC  
 Thr Tyr Asp Ile Val Asp Phe Ala Asn Gly Asn Ala Thr Thr Ala Thr Val Thr 3132 19/47

3159 CAT GAT ACC GCT AAC AAA ACC AGT AAA GTG GTA TAT GAT GTG AAT GTG GAT GAT  
 His Asp Thr Ala Asn Lys Thr Ser Lys Val Val Tyr Asp Val Asn Val Asp Asp 3186

3213 ACA ACC ATT CAT CTA ACA GGC ACT GAT GAC AAT AAA AAA CTT GGC GTC AAA ACC  
 Thr Thr Ile His Leu Thr Gly Thr Asp Asp Asn Lys Lys Leu Gly Val Lys Thr 3240

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## FIG.6 con't.

ACC AAA CTG AAC AAA ACA AGT GCT AAT GGT AAT ACA GCA ACT AAC TTT AAT GTT	3267	3294
Thr Lys Leu Asn Lys Thr Ser Ala Asn Gly Asn Thr Ala Thr Asn Phe Asn Val		
AAC TCT AGT GAT GAT GAA GAT GCC CTT GTT AAC GCC AAA GAC ATC GCC GAA AAT CTA	3321	3348
Asn Ser Ser Asp Asp Glu Asp Ala Leu Val Asn Ala Lys Asp Ile Ala Glu Asn Leu		
AAC ACC CTA GCC AAG AAG GAA ATT CAC ACC ACC AAA GGC ACA GCA GAC ACC GCC CTA	3375	3402
Asn Thr Leu Leu Ala Lys Glu Ile His Thr Thr Lys Gly Thr Ala Asp Thr Ala Leu		
CAA ACC TTT ACC GTT AAA AAG AAG GTA GAT GAA AAT AAT AAT GCT GAT GAC GCC AAC	3429	3456
Gln Thr Phe Thr Val Lys Lys Val Asp Glu Asn Asn Asn Ala Asp Ala Asn		

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FIG.6 con't.

3483  
 GCC ATC ACC GTG GGT CAA AAG AAC GCA AAT AAT CAA GTC AAC ACC CTA ACA CTC  
 Ala Ile Thr Val Gly Gln Lys Asn Ala Asn Asn Val Asn Thr Leu Thr Leu

3510  
 3537  
 AAA GGT GAA AAC GGT CTT AAT ATT AAA ACC GAC AAA AAT GGT ACG GTT ACC TTT  
 Lys Gly Glu Asn Gly Leu Asn Ile Lys Thr Asp Lys Asn Gly Thr Val Thr Phe

3564  
 3591  
 GGC ATT AAC ACC ACA AGC GGT CTT AAA GCC GGC AAA AGC ACC CTA AAC GAC GGT  
 Gly Ile Asn Thr Thr Ser Gly Leu Lys Ala Gly Ser Thr Leu Asn Asp Gly

3618  
 3645  
 GGC TTG TCT ATT AAA AAC CCC ACT GGT AGC GAA CAA ATC CAA GTC GGT GCT GAT  
 Gly Leu Ser Ile Lys Asn Pro Thr Gly Ser Glu Gln Ile Gln Val Gly Ala Asp

3672  
 3699  
 GGC GTG AAG TTT GCC AAG GTT AAT AAT AAT GGT GTA GGT GCT GGC ATT GAT  
 Gly Val Lys Phe Ala Lys Val Asn Asn Asn Gly Val Val Gly Ala Gly Ile Asp

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FIG.6 con't.

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GGC ACA ACT CGC ATT ACC AGA GAT GAA ATT GGC TTT ACT GGG ACT AAT GGC TCA	3753	3780
Gly Thr Thr Arg Ile Thr Arg Asp Glu Ile Gly Phe Thr Gly Thr Asn Gly Ser		
CTT GAT AAA AGC AAA CCC CAC CTA AGC AAA GAC GGC ATT AAC GCA GGT GGT AAA	3807	3834
Leu Asp Lys Ser Lys Pro His Leu Ser Lys Asp Gly Ile Asn Ala Gly Gly Lys		
AAG ATT ACC AAC ATT CAA TCA GGT GAG ATT GCC CAA AAC AGC CAT GAT GCT GTG	3861	3888
Lys Ile Thr Asn Ile Gln Ser Gly Glu Ile Ala Gln Asn Ser His Asp Ala Val		
ACA GGC GGC AAG ATT TAT GAT TTA AAA ACC GAA CTT GAA AAC AAA ATC AGC AGT	3915	3942
Thr Gly Gly Lys Ile Tyr Asp Leu Lys Thr Glu Leu Glu Asn Lys Ile Ser Ser		

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FIG.6 con't.

3969 3996  
 ACT GCC AAA ACA GCA CAA AAC TCA TTA CAC GAA TTC TCA GTA GCA GAT GAA CAA  
 Thr Ala Lys Thr Ala Gln Asn Ser Leu His Glu Phe Ser Val Ala Asp Glu Gln

4023 4050  
 GGT AAT AAC TTT ACG GTT AGT AAC CCT TAC TCC AGT TAT GAC ACC TCA AAG ACC  
 Gly Asn Asn Phe Thr Val Ser Asn Pro Tyr Ser Ser Tyr Asp Thr Ser Lys Thr

4077 4104  
 TCT GAT GTC ATC ACC TTT GCA GGT GAA AAC GGC ATT ACC ACC AAG GTA AAT AAA  
 Ser Asp Val Ile Thr Phe Ala Gly Glu Asn Gly Ile Thr Thr Lys Val Asn Lys

4131 4158  
 GGT GTG GTG CGT GTG GGC ATT GAC CAA ACC AAA GGC TTA ACC ACG CCT AAG CTG  
 Gly Val Val Arg Val Gly Ile Asp Gln Thr Lys Gly Leu Thr Thr Pro Lys Leu

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4185	ACC GTG GGT AAT AAT AAT AAT GGC AAA GGC ATT GTC ATT GAC AGC CAA AAT GGT CAA	4212
	Thr Val Gly Gly Asn Asn Asn Asn Lys Gly Ile Val Ile Asp Ser Gln Asn Gly Gln	
4239	AAT ACC ATC ACA GGA CTA AGC AAC ACT CTA GCT AAT GTT ACC AAT GAT AAA GGT	4266
	Asn Thr Ile Thr Gly Leu Ser Ser Asn Thr Leu Ala Asn Val Thr Asn Asp Lys Gly	
4293	AGC GTA CGC ACC ACA GAA CAG GGC AAT ATA ATC AAA GAC GAA GAC AAA ACC CGT	4320
	Ser Val Arg Thr Thr Thr Glu Gln Gln Gly Asn Ile Ile Lys Asp Glu Asp Lys Thr Arg	
4347	GCC GCC AGC ATT GTT GAT GTG CTA AGC GCA GGC TTT AAC TTT CAA GGC AAT GGT	4374
	Ala Ala Ser Ile Val Val Asp Val Val Asp Val Val Asp Ala Gln Gly Asn Gly	
4401	GAA GCG GTT GAC TTT GTC TCC ACT TAT GAC ACC GTC AAC TTT GCC GAT GGC AAT	4428
	Glu Ala Val Asp Phe Val Val Thr Thr Tyr Asp Thr Val Asn Asn Phe Ala Asp Gly Asn	

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FIG.6 con't.

4482  
 GCC ACC ACC GCT AAG GTG ACC TAT GAT GAC ACA AGC AAA ACC AGT AAA GTG GTC  
 Ala Thr Thr Ala Lys Val Thr Tyr Asp Asp Thr Ser Lys Val Val  
 4455

4536  
 TAT GAT GTC AAT GTG GAT GAT ACA ACC ATT GAA GTT AAA GAT AAA AAA CTT GGC  
 Tyr Asp Val Asn Val Asp Thr Thr Thr Ile Glu Val Lys Asp Lys Lys Leu Gly  
 4509

4590  
 GTA AAA ACC ACC ACA TTG ACC AGT ACT GGC ACA GGT GCT AAT AAA TTT GCC CTA  
 Val Lys Thr Thr Thr Leu Thr Ser Thr Gly Thr Gly Ala Asn Lys Phe Ala Leu  
 4563

4644  
 AGC AAT CAA GCT ACT GGC GAT GCG CTT GTC AAG GCC AGT GAT ATC GTT GCT CAT  
 Ser Asn Gln Ala Thr Gly Asp Ala Leu Val Lys Ala Ser Asp Ile Val Ala His  
 4617

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FIG.6 con't.

4671  
CTA AAC ACC TTA TCT GGC GAC ATC CAA ACT GCC AAA GGG GCA AGC CAA GCG AAC  
Leu Asn Thr Leu Ser Gly Asp Ile Gln Thr Ala Lys Gly Ala Ser Gln Ala Asn 4698

4725  
AAC TCA GCA GGC TAT GTG GAT GCT GAT GGC AAT AAG GTC ATC TAT GAC AGT ACC  
Asn Ser Ala Gly Tyr Val Asp Ala Asp Gly Asn Lys Val Ile Tyr Asp Ser Thr 4752

4779  
GAT AAC AAG TAC TAT CAA GCC AAA AAT GAT GGC ACA GTT GAT AAA ACC AAA GAA  
Asp Asn Lys Tyr Tyr Gln Ala Lys Asn Asp Gly Thr Thr Lys Lys Glu 4806

4833  
GTT GCC AAA GAC AAA CTG GTC GCC CAA GCC CAA ACC CCA GAT GGC ACA TTG GCT  
Val Ala Lys Asp Lys Leu Val Ala Gln Ala Gln Thr Pro Asp Gly Thr Leu Ala 4860

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## FIG.6 con't.

4887 CAA ATG AAT GTC AAA TCA GTC ATT AAC AAA GAA CAA GTA AAT GAT GCC AAT AAA  
 Gln MET Asn Val Lys Ser Val Ile Asn Lys Glu Gln Val Asn Asp Ala Asn Lys 4914

4941 AAG CAA GGC ATC AAT GAA GAC AAC GCC TTT GTT AAA GGA CTT GAA AAA GCC GCT  
Lys Gln Gly Ile 4968  
 Lys Glu Lys Ala Phe Val Lys Gly Leu Glu Lys Ala Ala

4995 TCT GAT AAC AAA ACC AAA AAC GCC GCA GTA ACT GTG GGT GAT TTA AAT GCC GTT  
 Ser Asp Asn Lys Thr Lys Asn Ala Ala Val Thr Val Gly Asp Leu Asn Ala Val 5022

5049 GCC CAA ACA CCG CTG ACC TTT GCA GGG GAT ACA GGC ACA ACG GCT AAA AAA CTG  
 Ala Gln Thr Pro Leu Thr Phe Ala Gly Asp Thr Gly Thr Thr Ala Lys Lys Leu 5076

5103 GGC GAG ACT TTG ACC ATC AAA GGT GGG CAA ACA GAC ACC AAT AAG CTA ACC GAT  
 Gly Glu Thr Leu Thr Ile Lys Gly Gly Gln Thr Asp Thr Asn Lys Leu Thr Asp 5130

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FIG.6 con't.

AAT AAC ATC GGT GTG GTA GCA GGT ACT GAT GGC TTC ACT GTC AAA CTT GCC AAA	5157	5184
Asn Asn Ile Gly Val Val Ala Gly Thr Asp Gly Phe Thr Val Lys Leu Ala Lys		
GAC CTA ACC AAT CTT AAC AGC GTT AAT GCA GGT GGC ACC AAA ATT GAT GAC AAA	5211	5238
Asp Leu Thr Asn Leu Asn Ser Val Asn Ala Gly Thr Lys Ile Asp Asp Lys		
GGC GTG TCT TTT GTA GAC TCA AGC GGT CAA GCC AAA GCA AAC ACC CCT GTG CTA	5265	5292
Gly Val Ser Phe Val Asp Ser Ser Gly Gln Ala Lys Ala Asn Thr Pro Val Leu		
AGT GCC AAT GGG CTG GAC CTG GGT GGC AAG GTC ATC AGT AAT GTG GGC AAA GGC	5319	5346
Ser Ala Asn Gly Leu Asp Leu Gly Gly Lys Lys Val Ile Ser Asn Val Gly Lys Gly		

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FIG.6 con't.

5373  
 ACA AAA GAT ACC GAC GCT GCC AAT GTA CAA CAG TTA AAC GAA GTA CGC AAC TTG  
 Thr Lys Asp Thr Asp Ala Ala Asp Val Gln Gln Leu Asn Glu Val Arg Asn Leu 5400

5427  
 TTG GGT CTT GGT AAT GGT AAT GAT AAC GCT GAC GGC AAT CAG GTA AAC ATT  
 Leu Gly Leu Gly Asn Ala Ala Gly Asn Asp Asn Ala Asp Gly Asn Gln Val Asn Ile 5454

5481  
 GCC GAC ATC AAA AAA GAC CCA AAT TCA GGT TCA TCT AAC CGC ACT GTC ATC  
 Ala Asp Ile Lys Lys Asp Pro Asn Ser Gly Ser Ser Ser Asn Arg Thr Val Ile 5508

5535  
 AAA GCA GGC ACG GTA CTT GGC GGT AAA GGT AAT AAC GAT ACC GAA AAA CTT GCC  
 Lys Ala Gly Thr Val Leu Gly Gly Lys Gly Asn Asn Asp Thr Glu Lys Leu Ala 5562

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## FIG.6 con't.

ACT GGT GGT ATA CAA GTG GGC GTG GAT AAA GAC GGC AAC GCT AAC GGC GAT TTA	5616
Thr Gly Gly Ile Gln Val Gly Val Asp Lys Asp Gly Asn Ala Asn Gly Asp Leu	5589
AGC AAT GTT TGG GTC AAA ACC CAA AAA GAT GGC AGC AAA AAA GGC CTG CTC GCC	5670
Ser Asn Val Trp Val Lys Thr Gln Lys Asp Gly Ser Lys Ala Leu Leu Ala	5643
ACT TAT AAC GCC GCA GGT CAG ACC AAC TAT TTG ACC AAC AAC CCC GCA GAA GCC	5724
Thr Tyr Asn Ala Ala Gly Gln Thr Asn Tyr Leu Thr Asn Asn Pro Ala Glu Ala	5697
ATT GAC AGA ATA AAT GAA CAA GGT ATC CGC TTC TTC CAT GTC AAC GAT GGC AAT	5751
Ile Asp Arg Ile Asn Glu Gln Gly Ile Arg Phe Phe His Val Asn Asp Gly Asn	5778
CAA GAG CCT GTG GTA CAA GGG CGT AAC GGC ATT GAC TCA AGT GCC TCA GGC AAG	5805
Gln Glu Pro Val Val Gln Gly Arg Asn Gly Ile Asp Ser Ser Ala Ser Gly Lys	5832

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FIG.6 con't.

5859  
 CAC TCA GTG GCG ATA GGT TTC CAG GCC AAG GCA GAT GGT GAA GCC GCC GGT GTC GCC  
 His Ser Val Ala Ile Gly Phe Gln Ala Lys Ala Asp Gly Glu Ala Ala Val Ala

5913  
 ATA GGC AGA CAA ACC CAA GCA GGC AAC CAA TCC ATC GCC ATC GGT GAT AAC GCA  
 Ile Gly Arg Gln Thr Gln Ala Gly Asn Gln Ser Ile Ala Ile Gly Asp Asn Ala

5967  
 CAA GCC ACG GGC GAT CAA TCC ATC GCC ATC GGT ACA GGC AAT GTG GTA GCA GGT  
 Gln Ala Thr Gly Asp Gln Ser Ile Ala Ile Gly Thr Gly Asn Val Val Ala Gly

6021  
 AAG CAC TCT GGT GCC ATC GGC GAC CCA AGC ACT GTT AAG GCT GAT AAC AGT TAC  
 Lys His Ser Gly Ala Ile Gly Asp Pro Ser Thr Thr Val Lys Ala Asp Asn Ser Tyr

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## FIG.6 con't.

6102  
 AGT GTG GGT AAT AAC AAC CAG TTT ACC GAT GCC ACT CAA ACC GAT GTC TTT GGT  
 Ser Val Gly Asn Asn Asn Gln Phe Thr Asp Ala Thr Gln Thr Asp Val Phe Gly

6156  
 GTG GGC AAT AAC ATC ACC GTG ACC GAA AGT AAC TCG GTT GCC TTA GGT TCA AAC  
 Val Gly Asn Asn Ile Thr Val Thr Glu Ser Asn Ser Val Ala Leu Gly Ser Asn

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6210  
 TCT GCC ATC AGT GCA GGC ACA CAC GCA GGC ACA CAA GGC GGC AAA TCT GAC GGC  
 Ser Ala Ile Ser Ala Gly Thr His Ala Gly Thr Gln Ala Lys Lys Ser Asp Gly

6264  
 ACA GCA GGT ACA ACC ACC ACA GCA GGT GCA ACC GGT ACG GTT AAA GGC TTT GCT  
 Thr Ala Gly Thr Thr Thr Ala Gly Ala Thr Gly Thr Val Lys Gly Phe Ala

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## FIG.6 con't.

6291 GGA CAA ACG GCG GTT GGT GCG GTC TCC GTG GGT GCC TCA GGT GCT GAA CGC CGT 6318  
 Gly Gln Thr Ala Val Gly Ala Val Ser Val Gly Ala Ser Gly Ala Glu Arg Arg

6345 ATC CAA AAT GTG GCA GCA GGT GAG GTC AGT GCC ACC AGC ACC GAT GCG GTC AAT 6372  
 Ile Gln Asn Val Ala Ala Gly Glu Val Ser Ala Thr Ser Thr Asp Ala Val Asn

6399 GGT AGC CAG TTG TAC AAA GCC ACC CAA AGC AGC ATT GCC AAC GCA ACC AAT GAG CTT 6426  
 Gly Ser Gln Leu Tyr Lys Ala Thr Gln Ser Ile Ala Asn Ala Thr Asn Glu Leu 33/47

6453 GAC CAT CGT ATC CAC CAA AAC GAA AAT AAG GCC AAT GCA GGG ATT TCA TCA GCG 6480  
 Asp His Arg Ile His Gln Asn Glu Asn Lys Ala Asn Ala Ala Gly Ile Ser Ser Ala

6507 ATG GCG ATG GCG TCC ATG CCA CAA GCC TAC ATT CCT GGC AGA TCC ATG GTT ACC 6534  
 MET Ala MET Ala Ser MET Pro Gln Ala Tyr Ile Pro Gly Arg Ser MET Val Thr

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## FIG.6 con't.

6561  
 GGG GGT ATT GCC ACC CAC AAC GGT CAA GGT GCG GTG GCA GTG GGA CTG TCG AAG  
 Gly Gly Ile Ala Thr His Asn Gly Gln Gly Ala Val Ala Val Gly Leu Ser Lys 6588

6615  
 CTG TCG GAT AAT GGT CAA TGG GTA TTT AAA ATC AAT GGT TCA GCC GAT ACC CAA  
 Leu Ser Asp Asn Gly Gln Trp Val Phe Lys Ile Asn Gly Ser Ala Asp Thr Gln 6642

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 6669  
 GGC CAT GTA GGG GCG GCA GTT GGT GCA GGT TTT CAC TTT TAA GCC ATA AAT CGC  
 Gly His Val Gly Ala Ala Val Gly Ala Gly Phe His Phe 6696

6723  
 AAG ATT TTA CTT AAA AAT CAA TCT CAC CAT AGT TGT ATA AAA CAG CAT CAG CAT 6750

6777  
 CAG TCA TAT TAC TGA TGC TGA TGT TTT TTA TCA CTT AAA CCA TTT TAC CGC TCA 6804

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FIG.6 con't.

6831 6858  
AGT GAT TCT CTT TCA CCA TGA CCA AAT CGC CAT TGA TCA TAG GTA AAC TTA TTG

6885 6912 35/47  
AGT AAA TTT TAT CAA TGT AGT TGT TAG ATA TGG TTA AAA TTG TGC CAT TGA CCA

6939 6966  
AAA AAT GAC CGA TTT ATC CCG AAA ATT TCT GAT TAT GAT CCG TTG ACC TGC AGG

TCG AC

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FIG. 7(a)

enzyme	1	1744	3487	5230	6973
ACCII	!				
AClNI	!	!			
AgeI				!	
Ama87I		!			
AseI			!		
AsnI			!		
AvaiI		!			
AvrII		!			
BcnI					!
BglI			!		
BlnI		!			
BpmI			!		
BsaWI				!	
Bsh1236I	!				
BsiWI				!	
Bsp143II		!			
BstH2I					
BstUI	!				
BstX2I					!

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FIG. 7(a). con't.

BstYI	---
CfrI	---
DraIII	---
EaeI	---
Eco32I	---
Eco88I	---
EcoNI	---
EcoRI	---
EcoRV	---
GsuI	---
HaeII	---
HindIII	---
KpnI	---
MfeI	---
MflI	---
MunI	---
MvnI	---
NciI	---
Pfl23II	---

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FIG. 7(a). con't.

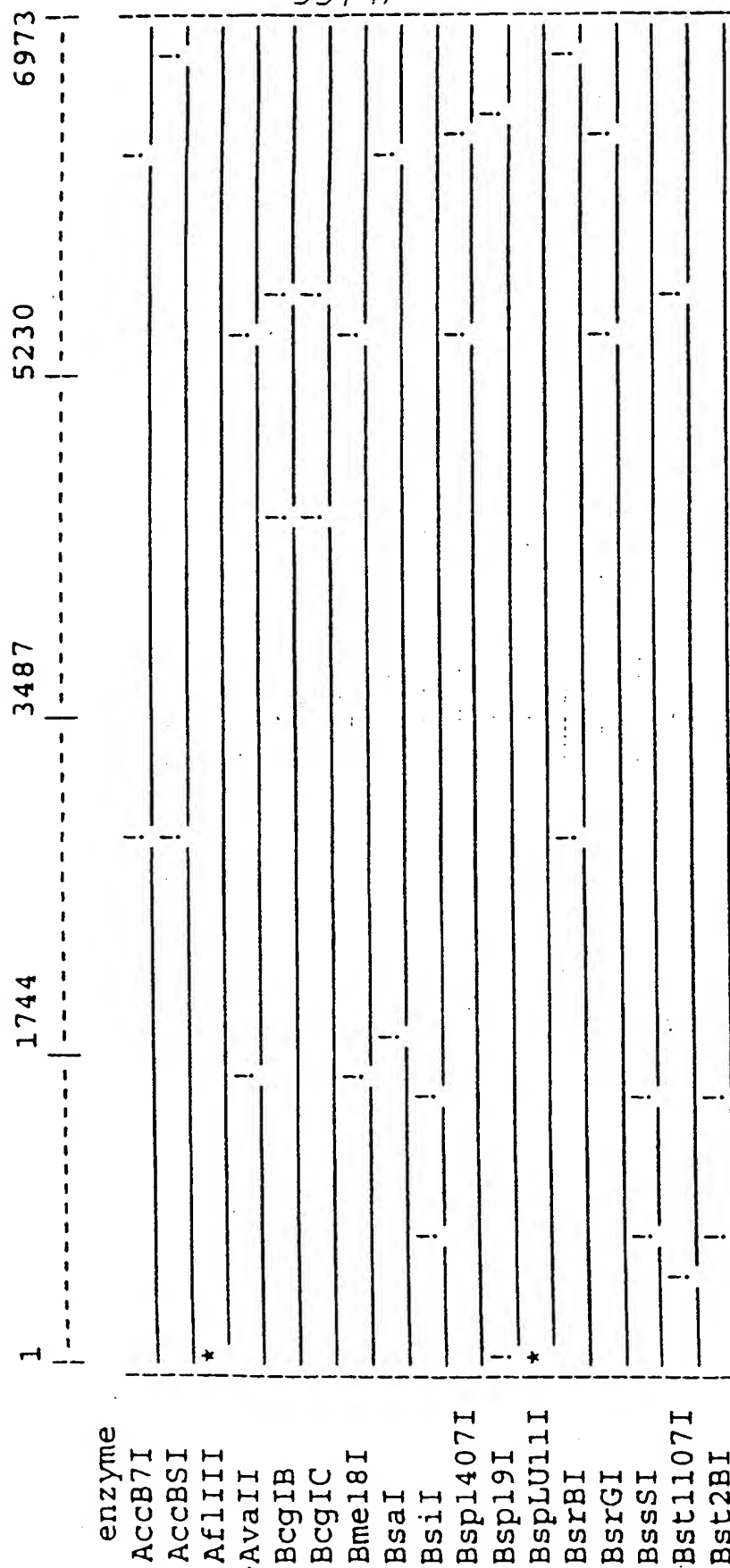
PinAI	
PspLI	
PvuII	
SaII	
SbfI	
Sfr274I	
SmiI	
SpeI	
SplI	
Sse8387I	
SunI	
Swal	
Thai	
VspI	
XbaI	
XhoI	
XhoII	

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FIG. 7(b).

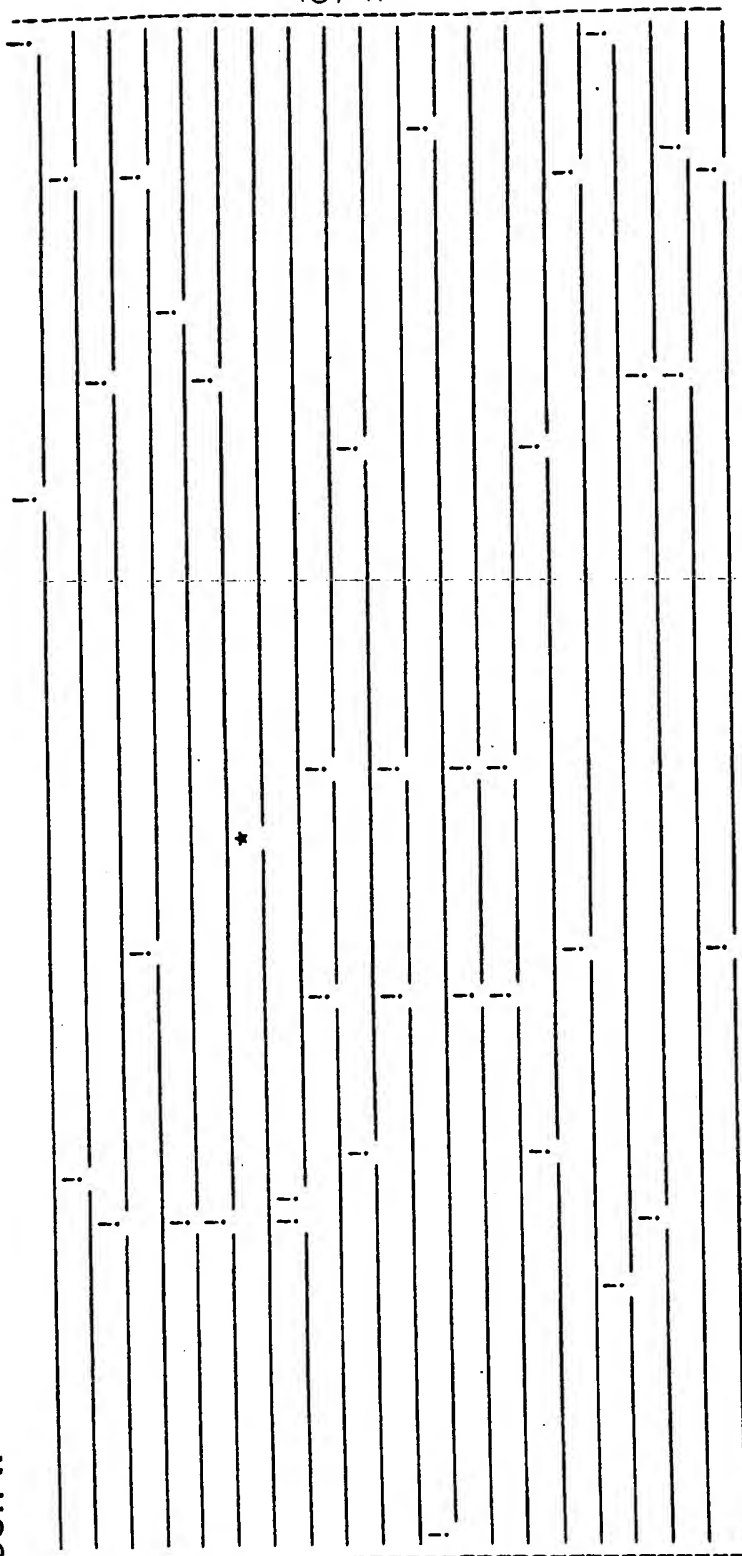


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FIG. 7(b). con't.

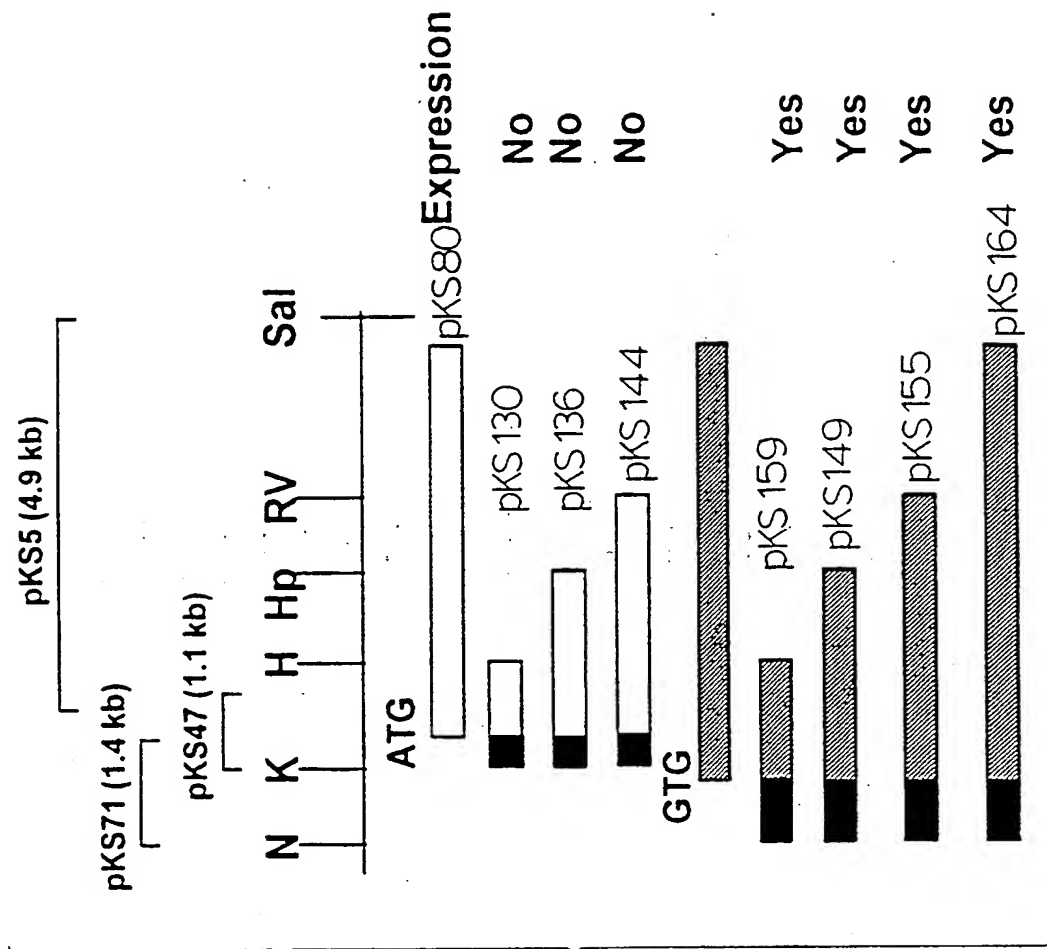
DrdI  
 Eco31I  
 Eco47I  
 Esp1396I  
 FauI  
 HgiEI  
 HpaI  
 MaeII  
 MroNI  
 MspAII  
 NaeI  
 NcoI  
 NgoAIV  
 NgoMI  
 NspBII  
 PflMI  
 PstI  
 SniI  
 SspBI  
 Van91I



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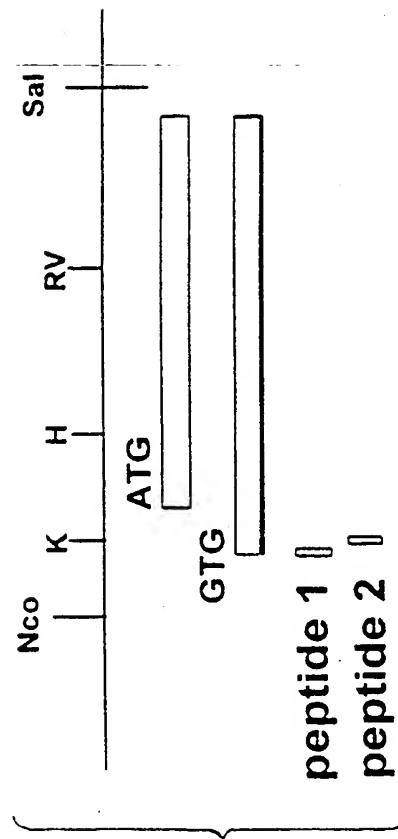
FIG.8.



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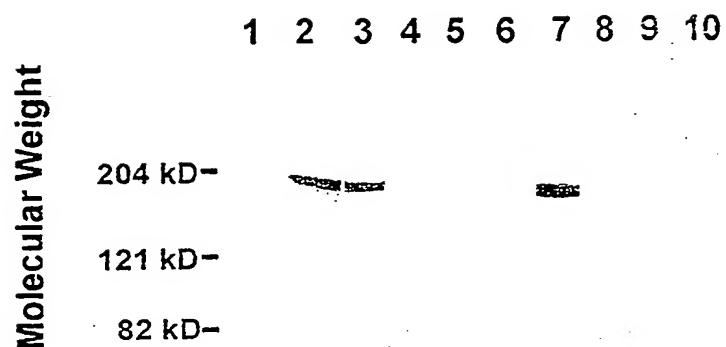
FIG.9.



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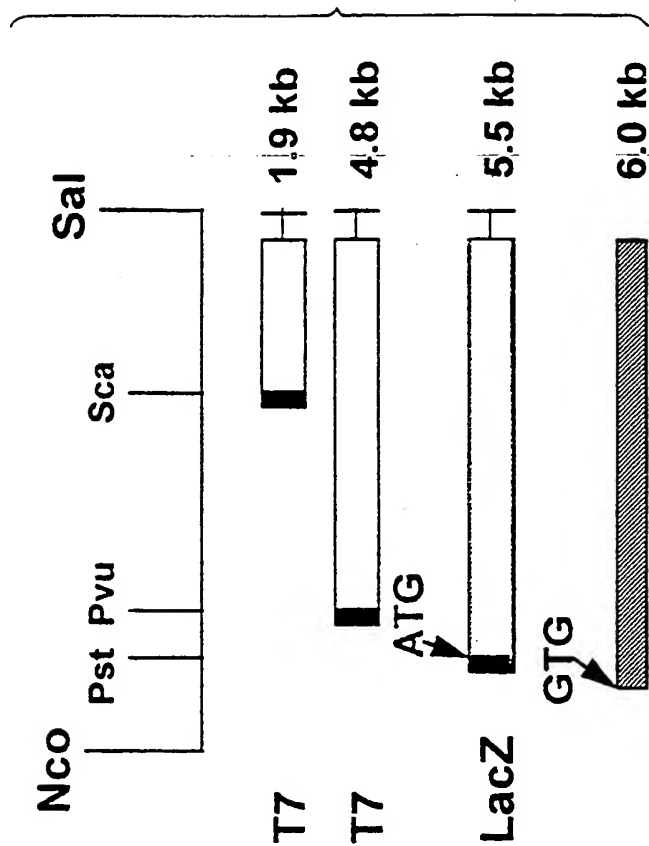
FIG.10.



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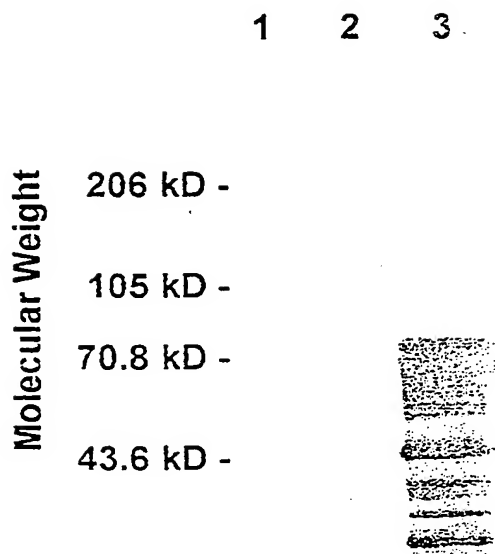
FIG.11.



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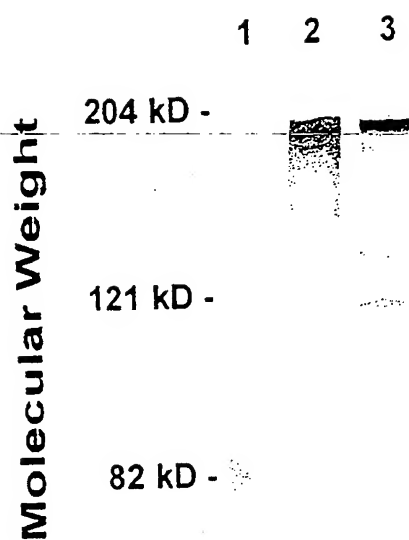
FIG.12.



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FIG.13.

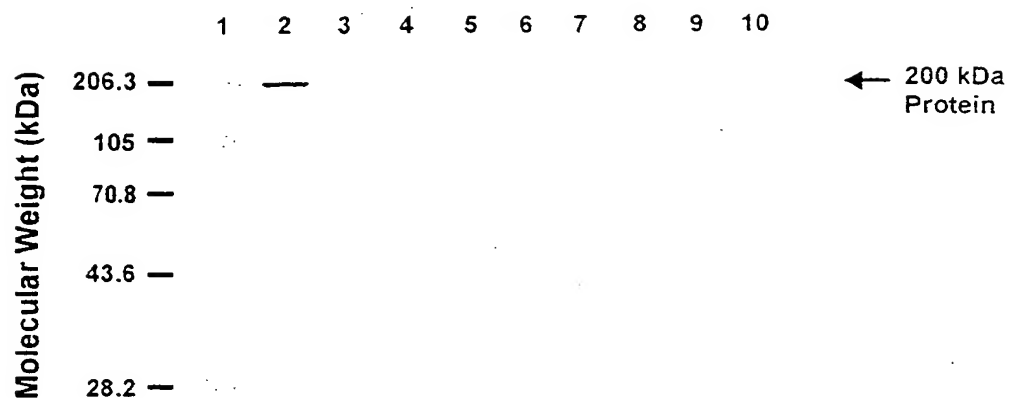


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FIG.14.



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A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 C12N15/31 C07K14/22 A61K39/095 C12N15/62 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 03761 (UNIV TEXAS) 4 March 1993	1-3, 10-12, 16,17, 20-26, 29-37
Y	see page 42, paragraph 2 - paragraph 3; claims 1-40; figures 4,5 see page 1, paragraph 1	18,19
Y	WO,A,93 10214 (GEORGIU GEORGE) 27 May 1993 see abstract; claims 1,2	18
Y	WO,A,91 09952 (CANADA MAJESTY IN RIGHT OF) 11 July 1991 see claims 1-15	19
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

11 September 1996

Date of mailing of the international search report

27.09.96

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